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Molecular Definition of a Region of Chromosome 21 That Causes Features of the Down Syndrome Phenotype

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Summary

Down syndrome (DS) is a major cause of mental retardation and heart disease. Although it is usually caused by the presence of an extra chromosome 21, a subset of the diagnostic features may be caused by the presence of only band 21q22. We now present evidence that significantly narrows the chromosomal region responsible for several of the phenotypic features of DS. We report a molecular and cytogenetic analysis of a three-generation family containing four individuals with clinical DS as manifested by the characteristic facial appearance, endocardial cushion defect, mental retardation, and probably dermatoglyphic changes. Autoradiograms of quantitative Southern blots of DNAs from two affected sisters, their carrier father, and a normal control were analyzed after hybridization with two to six unique DNA sequences regionally mapped on chromosome 21. These include cDNA probes for the genes for CuZn-superoxide dismutase (*SOD1*) mapping in 21q22.1 and for the amyloid precursor protein (*APP*) mapping in 21q11.2-21.05, in addition to six probes for single-copy sequences: *D21S46* in 21q11.2-21.05, *D21S47* and *SF57* in 21q22.1-22.3, and *D21S39, D21S42,* and *D21S43* in 21q22.3. All sequences located in 21q22.3 were present in three copies in the affected individuals, whereas those located proximal to this region were present in only two copies. In the carrier father, all DNA sequences were present in only two copies. Cytogenetic analysis of affected individuals employing R and G banding of prometaphase preparations combined with in situ hybridization revealed a translocation of the region from very distal 21q22.1 to 21qter to chromosome 4q. Except for a possible phenotypic contribution from the deletion of chromosome band 4q35, these data provide a molecular definition of the minimal region of chromosome 21 which, when duplicated, generates the facial features, heart defect, a component of the mental retardation, and probably several of the dermatoglyphic changes of DS. This region may include parts of bands 21q22.2 and 21q22.3, but it must exclude the genes *SOD1* and *APP* and most of band 21q22.1, specifically the region defined by *SOD1*, *SF57* and *D21S47.*

Introduction

Down syndrome (DS), a major cause of mental retardation and heart disease, is usually caused by the presence of an extra chromosome 21. In some cases, however, it is caused by the presence of only the distal half of chromosome 21, band q22 (Epstein 1986). This band has been called the "Down syndrome region," as defined by the presence of a subset of the major phenotypic features of the syndrome. These features include mental retardation, congenital heart disease, the characteristic facial appearance, and probably the hand anomalies and dermatoglyphic changes. Furthermore, there is some cytogenetic evidence that a duplication of only

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the subband 21q22.1 and perhaps 21q22.2 may generate most of these abnormalities (Poissonnier et al. 1976; reviewed in Epstein [1986]). The gene *SOD1,* for CuZn-superoxide dismutase, is the only cloned gene located in this region. Therefore, although its contribution to the DS phenotype is still unknown, it had assumed the role of being a "molecular marker" of DS.

Recently, we and others have described rare individuals with a "partial" DS phenotype who have an apparently normal karyotype but harbor a small amount of extra material from chromosome 21 (Huret et al. 1987; Korenberg et al. 1988*b*). Molecular analysis of these individuals has provided the opportunity to define the region of the chromosome responsible for the phenotypic features of Down syndrome. The conceptual basis for this approach has been extensively discussed (Epstein 1986, 1990). We now report a Japanese family with a chromosome 21 duplication (or "partial" trisomy 21) exhibiting many of the characteristic physical features of DS. These include the characteristic DS facies, congenital heart disease of the endocardial cushion type, and hand anomalies, in addition to other minor features. Mental retardation was also present. From our analysis of this family, we have significantly narrowed the region responsible for the listed physical components of the DS phenotype by first establishing the existence of partial trisomy 21, then by excluding the gene *APP* (amyloid precursor protein) from the trisomic region, and, finally, by excluding *SOD1* from the region of band 21q22.1 involved in the generation of the DS features (Korenberg et al. 1988*a,* 1988*b*). Moreover, by combining molecular characterization with in situ hybridization and high-resolution cytogenetic analysis, we have been able to narrow the responsible region of band 21q22.1 to such a degree that it is below the limit of cytogenetic detection. Finally, our findings exclude all known cloned genes and some random DNA sequences mapping in band 21q22.1 from the region of duplication defined by this family and establish the basis for specifying molecular markers for the region of chromosome 21 responsible for the phenotypic features of DS listed above.

Material and Methods

Molecular

The DNA sequence probes used in these investigations are all unique to human chromosome 21. FB68L is a cDNA probe corresponding to the 3′ end of the APP locus (Tanzi et al. 1987). The cDNA probe for *SOD1* was a gift from Y. Groner. Probes for *D21S39, D21S42, D21S43, D21S47,* and *SF57* are single-copy DNA sequences unique to chromosome 21 (Korenberg et al. 1987). Probes were isolated as DNA fragments by preparative gel electrophoresis and labeled by oligonucleotide priming to a specific activity of $2–5 \times 10^9$ cpm/microgram according to the manufacturer's specifications (Amersham, Arlington Heights, IL).

DNA was isolated (Korenberg et al. 1987) from lymphoblastoid cell lines derived from DS family members and from normal unrelated placenta used as a control. Each DNA was digested with the restriction enzyme *Eco*RI (Bethesda Research Laboratories) according to the manufacturer's directions, size separated by 1% agarose gel electrophoresis, and transferred to nylon membranes (Hybond; Amersham) by standard techniques (Maniatis et al. 1982). Multiple gels were run, each containing at least three lanes of DNA from a given family member and two to three lanes of control DNA. The nylon membranes were then treated as previously described (Korenberg et al. 1989) and hybridized simultaneously with two to five of the DNA sequences described above. The hybridization probe mix always included a probe for *D21S46* as an internal chromosome 21 reference probe. This sequence has been previously mapped to band 21q21 or proximal (Korenberg et al. 1987) and refined (Korenberg et al. 1986, 1989, 1990) to band 21q11.2-21.05. Both assignments were quite proximal to the cytogenetically defined duplicated region and, therefore, likely to be present in two copies in both DS patients and controls. This has been confirmed both by the present analysis and by subsequent experimental comparison to a chromosome 17 single-copy DNA sequence (data not shown).

Autoradiographs were generated by exposure of Kodak XAR film for the time required to bring the band signals to an approximately linear range of the film. This range was determined using a National Bureau of Standards penetrometer (data not shown). The results from each autoradiogram resulting from a single gel were analyzed separately to generate independent estimates of DNA sequence copy number, and the data from at least two autoradiograms were averaged and statistically analyzed.

Each lane of the autoradiogram was scanned by a Helena EDC densitometer, and the areas under each peak were integrated by computer. The copy number of each sequence is calculated as follows. First, the ratio of the density of the unknown band to that of band *D21S46* (the reference sequence) in the patient's DNA is calculated. Second, the same ratio is calculated in

the placental control DNA. Third, the standardized ratio, which represents the relative copy number of the unknown and reference sequences, is then calculated by dividing the ratio in the patient's DNA by the ratio in the control DNA. This is done separately for each lane of DNA, and the results are averaged for each patient across all lanes and statistically analyzed by the one-tailed *t*-test.

Cytogenetics and In Situ Hybridization

The probe for DNA sequence *D21S39* was radiolabeled by nick translation as described in Magenis et al. (1985) to a specific activity of 3.75×10^7 dpm/μg using [^{3}H]dTTP (65 Ci/mmol) and [^{3}H]dCTP (60 Ci/mmol; Amersham).

Lymphoblastoid cells from the father (III-1) and affected individuals IV-1 and IV-2 were synchronized with amethopterin to obtain an adequate number of cells in early metaphase (Yunis and Chandler 1977). Chromosome analyses were performed using trypsin G-banding and fluorescent R-banding with chromomycin A_3/distamycin A (Schweizer 1980).

In situ hybridization, silver-grain analysis, and photography were done as described elsewhere (Magenis et al. 1985). After development, the slides were R-banded using a modification (Magenis et al. 1985) of the technique of Schweizer (1980).

Results

Clinical

The pedigree of the three-generation Japanese family in which DS is segregating and photographs of the four affected individuals are shown in figures 1 and 2,

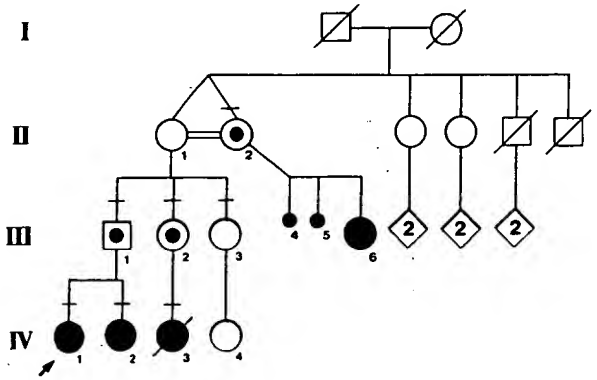


Figure 1 Pedigree of family with Down syndrome. ♂/□ indicates individuals studied; ○/⊡ indicates balanced translocation t(4:21) carriers; ●/■ indicates Down syndrome patients.

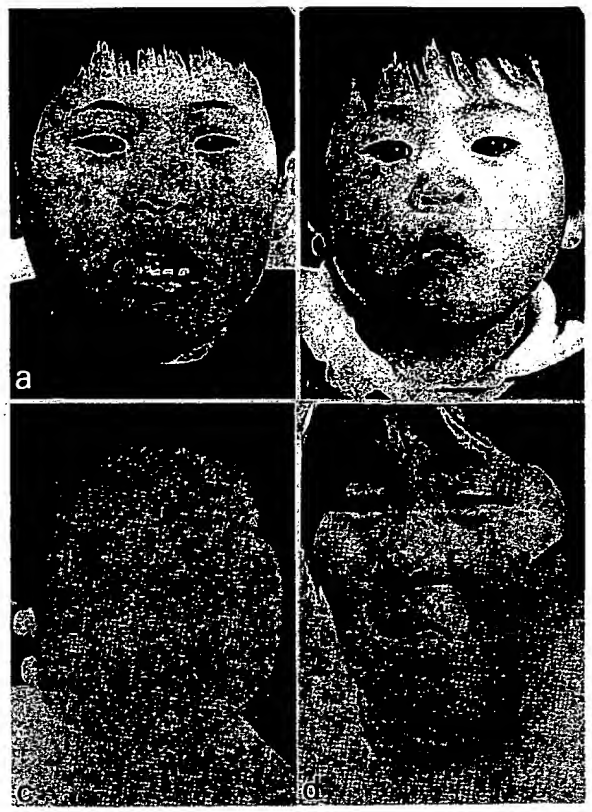


Figure 2 Photographs of the four affected individuals. *a*, Patient IV-1 at 6 1/2 years; *b*, patient IV-2 at age 2 years; *c*, patient IV-3 at 1 mo; *d*, patient III-6 at 30 years.

respectively, and the clinical findings are summarized in table 1. Taken together, the four patients exhibit the characteristic facial features of DS and are retarded; two have congenital heart disease, and two have most of the characteristic dermatoglyphic findings. One of the four had a history of hypotonia.

Cytogenetics and Molecular Analyses

Initial high-resolution G-banding analysis of the chromosomes of IV-1 and IV-2 revealed an apparently normal karyotype, with two normal chromosomes 21. However, high-resolution cytogenetic analysis of the father (III-1) of patients IV-1 and IV-2, and of the mothers of patients IV-3 and III-6 (fig. 1), revealed an abnormality of chromosome 21 in the region 21q22.1-22.2 in a karyotype that otherwise appeared normal.

To define the genetic content of the potentially duplicated region of chromosome 21 in these patients and its familial transmission, we performed a molecular analysis of the DNA of IV-1 and IV-2 and of their clini-

Table 1

Features of Affected Individuals

	IV-1	IV-2	IV-3	III-6
Age at evaluation (years)	6.5	2	Death at .5	34
Microcephaly	−0.9 SD	−1.8 SD		
Flat occiput	−			
Hypotonia	−	−	+	
Lax ligaments	+	+		
Poor suck at birth	+			
Delayed milestones	Motor speech	Speech		
Short stature	−1.8 SD	−1.4 SD		
Failure to thrive	+	−		
IQ	42 (at 6 years)		Retarded	
Dementia	−	−		−
Flat facies	+	+	+	+
Upslanted palpebral fissures	+	+	+	+
Epicanthic folds	+	+	+	+
Telecanthus	+	+		
Flat nasal bridge	+	+	+	+
Dentition abnormal	+			
Macroglossia	+	+		
High palate	−			
Open mouth	+			
Ears cupped or low set	−	−		
Short neck	−			
Heart disease	ASD		ECD	
Broad hands	+			
Brachydactyly	+	+		
Fifth-finger clinodactyly	+			
Dermatoglyphics:				
Finger pads	10 UL	3 UL, 7 W		
Third interdigital loops	+	+		
Hypothenar patterns	Bilateral	Bilateral		
Distal axial triradius (t")	+(left)	Bilateral		
Single transverse palmar crease	Bridged left crease			
Hallucal fibular loop	Bilateral	Bilateral		
First interdigital loop	Bilateral	Bilateral		
Sole open field patterns	Bilateral	Bilateral		

NOTE.—Presence (+) or absence (−) of each feature is noted as indicated. Features for which no information is available are indicated by blank spaces. ASD = atrial septal defect; ECD = endocardial cushion defect; UL = ulnar loop; W = whorl.

cally normal father. For this purpose we used a fine structure molecular map of chromosome 21 constructed by isolating 13 single-copy DNA sequences unique to chromosome 21 and establishing their regional location using a somatic cell hybrid and then a panel of DNAs from cells aneuploid for only parts of chromosome 21 (Korenberg et al. 1986, 1987, 1989; J. R. Korenberg and T. Falik-Borenstein, unpublished data).

Figure 3 shows four segments of the map, which are represented by eight DNA sequences. To analyze the extent of the suspected chromosome 21 duplication in the family being studied, we investigated the copy number of each of these sequences. *D21S46* and *APP* are located in 21q11.2-q21.05, outside the previously defined classical DS phenotype region, and *APP* is located below *D21S46* (Korenberg et al. 1987, 1989; Patterson

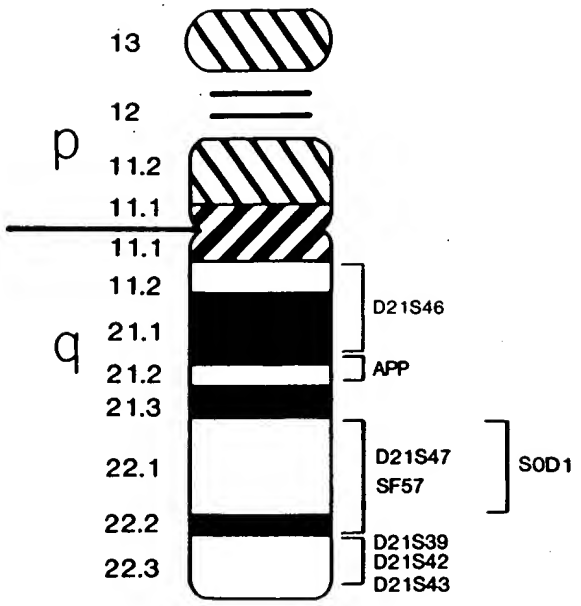


Figure 3 Physical map of chromosome 21 showing location of the chromosome 21–specific DNA sequences analyzed in the present study.

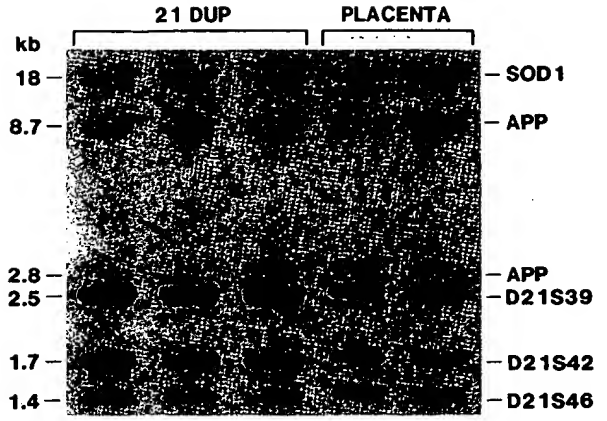


Figure 4 Autoradiogram of DNAs from patient IV-1 and from a control hybridized to chromosome 21 DNA sequences. The three lanes on the left, labeled 21 DUP, each contain DNA from this patient, and the two on the right contain control DNA. Probe names are indicated at the right and *Eco*RI fragment sizes are indicated on the left of the autoradiogram.

et al. 1988); *SOD1* maps to the region including 21q22.1 (Sinet et al. 1976). Two single-copy random DNA sequences, *SF57* and *D21S47,* also map to the region including band 21q22.1 through proximal q22.3 (Korenberg et al. 1987; J. R. Korenberg and T. Falik-Borenstein, unpublished data). Finally, *D21S39, D21S42,* and *D21S43* are located in mid–band 21q22.3 (J. R. Korenberg and T. Falik-Borenstein, unpublished data).

We determined the relative copy number of specific DNA sequences using quantitative Southern blot dosage analyses as described above. Figure 4 presents an illustrative autoradiogram used for the analysis of DNA sequences *D21S46* (the reference sequence), *D21S47, SF57, D21S42,* and *D21S39.* Table 2 shows the results of the statistical analysis of the results obtained with these sequences, as well as *D21S43* and the genes *SOD1* and *APP.* The copy number of each DNA sequence is presented along with the standardized ratio obtained for DS patients IV-1 and IV-2 and their normal father

Table 2

Chromosome 21 DNA Sequence Copy Number

Map Position and DNA Sequence	Mean Ratio (±SE)			Copy Number		
	IV-1	IV-2	Carrier Father	IV-1	IV-2	Carrier Father
Q11.2–21.05:						
FB68L (*APP*)	.91 (.044)	1.0	.8	2	2	2
Q22.1:						
SOD1	1.01 (.034)	1.1	.9	2	2	2
D21S47.....	.93 (.04)	NT	NT	2	...	...
SF57	.94 (.07)	NT	NT	2	...	...
Q22.3:						
D21S39.....	1.53 (.035)*	1.8**	1.1	3	3	2
D21S42.....	1.6 (.04)*	1.74**	1.1	3	3	2
D21S43.....	1.83 (.16)*	NT	NT	3	...	...

NOTE.—NT = not tested.
*Different from 1.0 by *t*-test; $P < .0001$.
**Different from 1.0 by *t*-test; $P < .05$.

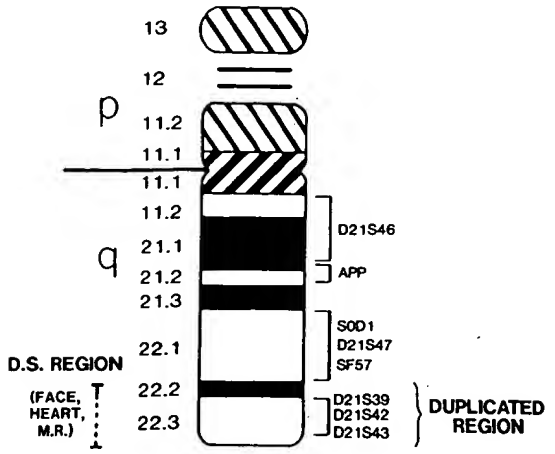
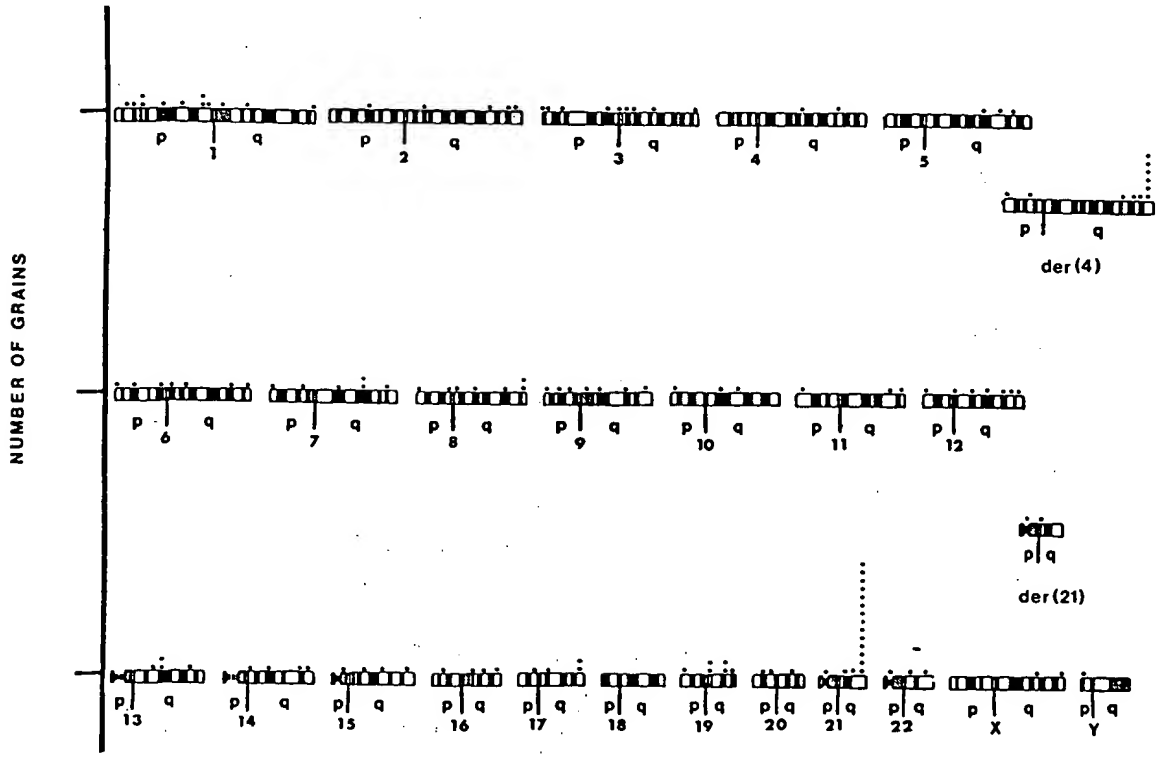


Figure 5 Physical map of chromosome 21 showing location of DS phenotypic region defined by the present study.

III-1. In the two affected individuals, *D21S39, D21S42,* and *D21S43* are present in three copies, whereas *SF57, D21S47, SOD1,* and *APP* are present in only two. Therefore, as summarized in figure 5, in this family there is a duplication which includes bands 21q22.2 and 21q22.3 but excludes the region of band 21q22.1 defined by our probes. *SOD1, APP,* and the sequence *D21S46* have been excluded from the region of the duplication. CuZn-SOD enzyme activities in red blood cells were found to be normal in IV-1 and IV-2 and in their parents (data not shown).

To detect the presumed translocated segment, *in situ* hybridization was performed on cells from DS patients IV-1 and IV-2 and from their normal father III-1, using the probe for *D21S39* which we had shown to be in the duplicated region. The results shown in figure 5 confirmed the presence of a balanced translocation between chromosomes 4 and 21 in the normal father (III-1). A low background and the presence of all grains only over chromosomes permitted accurate assignment of map position. Of the total grains observed in a single individual, no chromosomal band had more than a single overlying grain except the following. In 100 cells examined from the father (fig. 6) there were 136 grains, of which a significant proportion of grains were seen on the normal chromosome 21 (14% of the cells; 10% of the grains) and at the telomeric region of the derivative chromosome 4 (6% of the cells; 4.3% of the grains), but not on the derivative chromosome 21 or the normal 4, suggesting the presence of 4;21 translo-



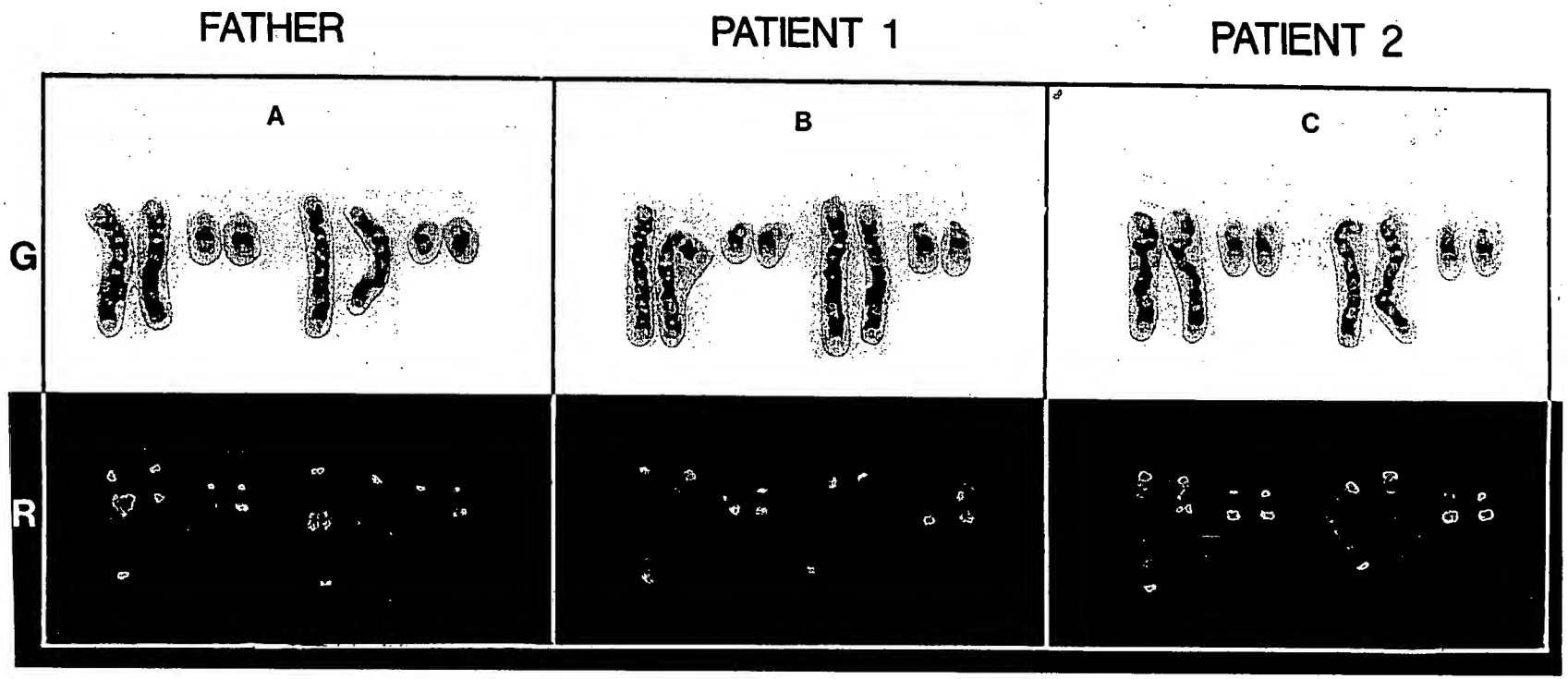


Figure 7 Homologous chromosome pairs 4 (*left*) and 21 (*right*) from patients IV-1 (*B*) and IV-2 (C) and from their normal carrier father, III-1 (*A*), shown with G-banding (*upper panels*) and R-banding (*lower panels*) at the ~550 band stage. The father's R-banded chromosomes are shown on the left lower panel. It is clear that the highly fluorescent tip of the long arm is present only on one, not both, of the chromosomes 21, and that the normally dull tip of 4q is now highly fluorescent. In contrast, the two patients' R-banded chromosomes shown in the middle and right panels reveal brightly fluorescent tips on both 21s in addition to one of the 4s. Although difficult to appreciate, all of band 21q22.2 is absent from the abnormal chromosome 21, presumably translocated to chromosome 4, and the tip is replaced by a dull band. Further, the tip of chromosome 4q is abnormal with the telomeric G band shown on the right of each pair replaced by a longer R band, originating from chromosome 21. Further, the proximal G band, 4q34, appears larger but single, presumably the result of the apposition of the chromosome 21 G band 21q22.2. The patients each carry two normal chromosomes 21 and one normal chromosome 4 plus the derivative 4 in contrast to their father, who carries only one normal chromosome 21, one normal chromosome 4, and both the derivative 21 and derivative 4 in the balanced state.

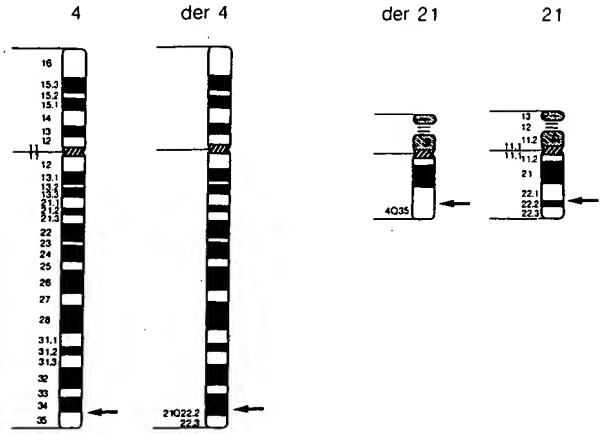


Figure 8 GBG-banded ideogram of the 4;21 translocation shown in fig. 7. Arrows indicate the breakpoints on distal chromosome 21q22.1 at the 21q22.2 border and on chromosome 4q35 proximal.

cation. In 100 cells examined from IV-1, there were 119 grains found with 8 grains (7% of grains) over chromosomes 21 and 6 grains (5%) over the derivative 4. Similarly, in 80 cells examined from IV-2, there were 111 grains found. Fifteen of these were over the distal 21 (18.8% of cells; 13.5% of grains), and 12 (15.0% of cells; 10.8% of grains) were on the distal tip of the derivative 4. These findings indicated the presence of a 4;21 translocation chromosome in addition to two normal chromosomes 21. The presence of the 4;21 translocation was confirmed with R-banding, and the breakpoints were defined with high-resolution G-banding as being in very proximal 4q35 and in distal 21q22.1 at the 21q22.2 border (Harnden and Klinger 1985) (figs. 7 and 8). At the level of resolution employed (about 550 bands/haploid genome), the unitary nature of the distal G-band, 4q34, suggests that the apposed break in chromosome 21 was below the limit of cytogenetic detection in distal 21q22.1 or in 21q22.2 as schematized in figure 8. Therefore, affected individuals, in addition to having a duplication of distal chromosome 21q, have a deletion of 4q35.

Discussion

We have described a three-generation family segregating four individuals with the phenotypic features of DS, three of them shown to be associated with a chromosome 21 rearrangement and the fourth untested. Using quantitative Southern blotting we first established that the duplicated region excludes DNA sequences mapping in 21q21 (*APP, D21S46*) and in 21q22.1 (*SOD1* and probably *D21S47, SF57*), but includes DNA sequences likely mapping in band 21q22.3 (*D21S39, D21S42, D21S43*). Using *D21S39* as a probe for in situ hybridization, we next established the existence of a 4;21 translocation, carried in the balanced state in intervening relatives in the pedigree, but in the unbalanced state in two of the individuals with phenotypic DS, resulting in partial trisomy 21. High-resolution cytogenetic analysis then excluded most of band 21q22.1 from the duplicated region, greatly narrowing the chromosome 21 region responsible for the facial features, heart defects, and probably the dermatoglyphic changes characteristic of DS seen in this family.

The Down Syndrome Region

The complete panoply of phenotypic features seen in Down syndrome (Epstein 1986) includes a large set of physical and biochemical features, but a subset of these is of obvious diagnostic, clinical, and social significance, namely, the facial appearance, congenital heart disease, and mental retardation, respectively. Previous work has shown that this subset of features may be produced by duplication including only band 21q22, as initially suggested by Niebuhr (1974), and perhaps only band 21q22.1-21q22.2 for the facial appearance and mental retardation (Poissonnier et al. 1976; Summitt 1981; Epstein 1986; Park et al. 1987). The importance of the 21q22 region for the production of the classical facial features of DS is emphasized by the lack of these features in individuals partially trisomic for other regions of chromosome 21. For example, duplication of 21pter-proximal q21 may be associated with a normal phenotype (Daniel 1979). Similarly, duplication of 21pter-q21 (likely of a larger region than the former case) does not appear to produce the characteristic DS subset of features but, rather, a different subset that shares only mental retardation in common, in addition to marked microcephaly, short stature, and hypoplastic nails (Park et al. 1987). Although these features may occasionally be a part of the DS phenotype, they are not constant or specific (Jackson et al. 1976) and therefore have not contributed significantly to the diagnosis of DS.

The precise assignment of a phenotypic feature to a particular region of chromosome 21 is limited by the resolution of cytogenetic analysis. Moreover, phenotypic mapping is made more difficult by the variability of phenotype of individuals with full trisomy 21. For example, only 40% of such cases have congenital heart disease. Indeed, in the published literature there is only

one case of partial trisomy 21 with congenital heart disease (Miyazaki et al. 1987). This variable expressivity is particularly important in that it limits phenotypic mapping power, because a phenotypic feature may be mapped by its presence but not excluded by its absence. In addition, cases of partial trisomy are rare, frequently associated with unbalanced translocations, and, therefore, usually accompanied by other aneuploidy which complicates the assignment of a particular feature to a region of chromosome 21. Consequently, although there is general agreement on the necessary involvement of band 21q22 in generating the characteristic facial appearance, mental retardation, and heart disease, there is little information on further subdivision of this region.

Our study has circumvented many of the above problems by the combined clinical, cytogenetic, and molecular analysis of a family with four cases of DS, all presumed to have the identical duplicated region. Although the affected members exhibit the phenotypic variability characteristic of trisomy 21, taken together they exhibit a broad subset of clinical DS features. We have, therefore, physically mapped the features seen in this family to the duplicated region that clearly involves 21q22.2-q22.3 and may include very distal 21q22.1, below the limit of cytogenetic detection. The features so mapped include the facial appearance (flat facial profile, upslanting palpebral fissures, epicanthal folds, flat nasal bridge, open mouth, protruding tongue), congenital heart defect of the endocardial cushion type, clinodactyly of the fifth finger, and probably the short fingers and dermatoglyphic changes (table 1). Neonatal hypotonia was noted only in patient IV-3, and this may have been due in part to her severe cardiac failure. This feature was, therefore, not mapped to the region under consideration.

These results are in agreement with those of McCormick et al. (1989) and may include regions in common with but below the limit of cytogenetic resolution of the patient of Poissonier et al. (1976), as analyzed in Rahmani et al. (1989). Both cases have some features of DS. However, study of the present family has also afforded an opportunity to define the chromosomal region responsible for the congenital heart disease in addition to the other phenotypic features of DS.

The necessary deletion (partial monosomy) of 4q35-4qter may also contribute to the phenotype seen in this family, particularly to the mental retardation. However, this deleted region is far smaller than any 4q deletion previously discussed. All of the three cases reported included a larger region, 4q32-qter (Fryns et al. 1981) or 4q33-qter (Mitchell et al. 1981; Stamberg et al. 1982), than that deleted in our family. These individuals had minor physical abnormalities (short nose, small mouth, cleft palate, small lower jaw, and limb abnormalities) in addition to *mild* developmental delays. Further, none of these had cardiac defects. Moreover, of the two reported patients in whom formal testing were done, both were of relatively high mental function. One had an IQ of 83–88 (within the low normal range), and the other, the one with the large 4q32-qter deletion, had an IQ of 68. Finally, although cardiac defects are seen with much larger deletions of 4q, none are of the endocardial cushion type seen in our family and characteristic of DS (reviewed in Mitchell et al. [1981]). These observations suggest that, although the 4q35 deletion in our patients may contribute to their physical features, both the endocardial cushion defect and some, though not necessarily all, of the mental impairment seen in our patients is produced by the duplication of chromosome 21. Finally, it is unlikely that genetic position effects of genes at the chromosomal breakpoints contribute significantly to the DS phenotype in this family, since the balanced translocation carriers are phenotypically normal.

For cytogenetic diagnostic purposes, it is important to note that the presence of a chromosome 21 translocation was strongly suspected from the pedigree and from the abnormal 21 observed in the normal father. However, it was still possible that the abnormal chromosome 21 carried an internal rearrangement predisposing to secondary meiotic duplication events that resulted in offspring with the appearance of DS. Indeed, no other karyotypic abnormality was detected by high-resolution G-banding until chromosome 4 was found to be abnormal by in situ hybridization and R-banding. This difficulty arises when translocations involve approximately equal pieces with similar banding patterns. In such cases, fluorescent R-banding may allow a differential degree of fluorescence among R-bands that may permit easy definition of a rearrangement. This caveat is well illustrated by our cases and has been emphasized in previous cases of 4;21 translocations (Dutrillaux et al. 1973; Mattei et al. 1981).

From our study we can conclude that the overexpression of both *APP* and *SOD1* is not necessary for the development of the phenotypic features seen in this family, including some elements of mental retardation. However, it is quite possible that the overexpression of *APP* contributes both to the neuropathology found in older individuals with trisomy 21 and to the dementia that sometimes results. Similarly, it is clear from ex-

periments with the *SOD1* transgenic mouse (Epstein et al. 1987) that the overexpression of *SOD1* may significantly alter both the expression of neurotransmitters (Elroy-Stein and Groner 1988) and neural histology (Avraham et al. 1988). Therefore, the potential importance of genes outside the molecular region defined in this report in the genesis of the overall phenotype of DS resulting from complete trisomy 21 cannot be underestimated (Epstein 1990). Nothing reported here is intended to imply that the region of duplication defined in the family being reported constitutes the entire segment of chromosome 21 responsible for the complete phenotype of DS. However, clinical and molecular investigation of such individuals should provide clues to defining the genetic basis of their phenotypes.

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Protocols to Establish Genotype-Phenotype Correlations in Down Syndrome

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Introduction

Progress in the genetic and physical mapping of chromosome 21 has now reached the point at which it is possible to begin the correlation of the phenotypic components of Down syndrome with imbalance of specific regions of the components of the chromosome. Several preliminary efforts in this direction have already been made and suggest that the phenotypic and molecular analysis of relatively rare individuals with chromosome 21 duplications ("partial trisomy") can be used to specify which regions of chromosome 21 are involved in the generation of specific components of the phenotype (see below). The ultimate goal of correlating genotype with phenotype (phenotypic mapping) is to make it possible to discover which particular genes are responsible for which aspects of the phenotype, thereby permitting the pathogenesis of the syndrome to be elucidated and, it is hoped, its most serious consequences to be prevented or ameliorated.

To facilitate the process of making a phenotypic map of Down syndrome, a workshop was sponsored by the National Institute of Child Health and Human Development on April 24–25, 1990, to develop protocols for obtaining and recording the necessary phenotypic, cytogenetic, and molecular data. It is anticipated that these protocols will provide the basis for analyzing and comparing the phenotypes and genotypes of individuals with different duplications involving the long arm of chromosome 21. The protocols which have been developed were designed to provide a uniform and precise specification of the phenotype and degree of chromosome imbalance of each individual to be studied. Although they are not intended for use in the diagnosis or investigation of Down syndrome per se, these protocols should nonetheless prove useful for these purposes and possibly for the care of persons with Down syndrome.

Rationale for Making Genotype-Phenotype Correlations from Analysis of Chromosome Duplications

The theoretical basis for phenotypic mapping of components of aneuploid syndromes has already been presented in detail (Epstein 1986, 1990). In brief,

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analysis of a wide variety of such syndromes has demonstrated that different aneuploid phenotypes are specific and are distinguishable from one another in so far as overall patterns of features are concerned. From this it has been inferred that the aneuploid phenotypes are, in the main, determined by the specific genes present in the region of imbalance. Although stochastic, environmental, and other genetic factors may influence the phenotype in any individual case, they are not the determinants of the phenotype. Furthermore, comparisons of syndromes resulting from overlapping duplications or deletions or from double aneuploidy (involving two different chromosomes) have shown that it is frequently possible to attribute individual components of a phenotype to imbalance of a particular chromosomal region. These observations, as well as recent work on small-deletion or contiguous-gene syndromes, indicate that individual phenotypic features, particularly those which are quite distinctive, are likely to be due to imbalance of one or a very few genes and should therefore be mappable. These observations also suggest that aneuploid phenotypes resulting from the presence or absence of a whole chromosome are not the result of imbalance of a single gene or of a single narrowly defined critical region. It is likely that a global component of the phenotype, such as mental retardation, will have many genetic determinants, even on a single unbalanced chromosome. However, it is possible that some determinants may have a more powerful influence than others or may determine a specific aspect of the retardation and will, therefore, be susceptible to analysis.

Current Status of Genotype-Phenotype Correlations in Down Syndrome

It is now over 30 years since Down syndrome was found to be caused by trisomy 21, and more than 15 years have elapsed since the role of band q22 in causing the phenotype of Down syndrome was suggested. Two changes in direction have recently been defined. First, it is now clear that genes in other regions contribute significantly to the phenotype. Second, the emergence of the physical map of chromosome 21 has eliminated the uncertainty of cytogenetic analyses and has made possible the molecular definition of regions responsible for specific phenotypic features of Down syndrome. A phenotypic map of Down syndrome based on *cytogenetic* analyses is shown in figure 1. This map, constructed from 17 well-defined cases of chromosome 21 duplications published since 1973, shows the overlaps of duplicated regions which are associated with the feature(s) indicated. The map must, for two reasons, be considered as indicating only the *minimal* regions involved in producing a particular feature. Too little information derives from small duplications, and the data are incomplete with regard to the *lack* of features in many of the patients. Furthermore, such analyses do not indicate the number of genes involved.

The phenotypic map of Down syndrome based on molecular analysis of chromosome 21 duplications is now emerging from studies conducted by a number of groups using the techniques detailed below. There is general consensus that, although the facial features of Down syndrome may be determined by genes in the

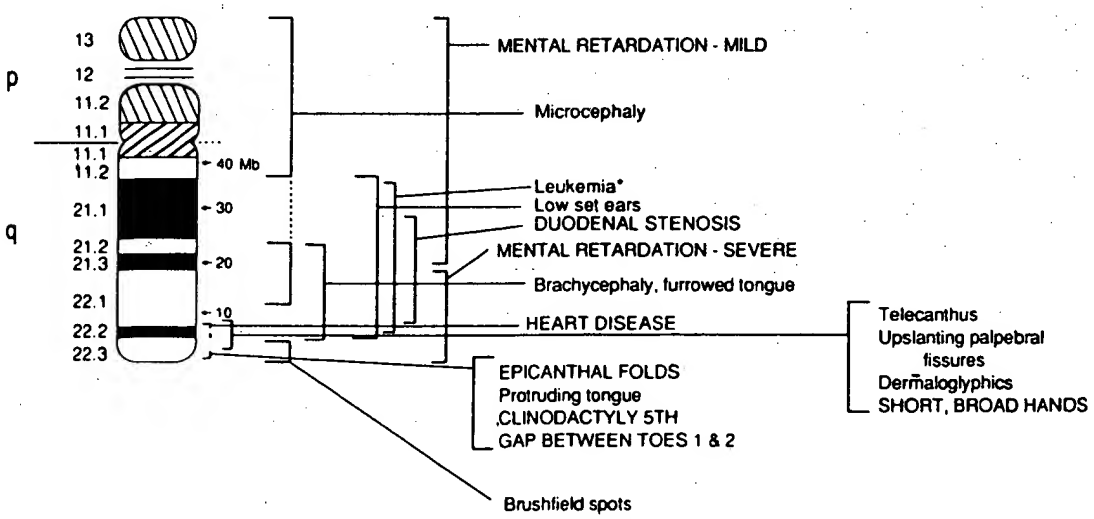

Figure 1 Down syndrome phenotypic map based on cytogenetic analysis, 1973–89 (Korenberg 1991)

region of the DNA marker *D21S55*→21qter (Korenberg et al. 1988, 1990; McCormick et al. 1988, 1989; Rahmani et al. 1989), the mental retardation results from imbalance of genes mapping throughout the chromosome. One group has suggested that the region around *D21S55,* between D21S17 and *ETS2,* probably contains genes contributing significantly to the pathogenesis of some of the facial features (flat nasal bridge, macroglossia, folded ears), incurved fifth fingers, gap between first and second toes, hypotonia, and mental retardation (Rahmani et al. 1990). Moreover, the minimal regions likely to contain the gene(s) determining the congenital heart disease and the duodenal stenosis have also been defined, as *D21S55*→21qter (Korenberg et al. 1988, 1990) and *D21S8*→*D21S15* (Korenberg et al. 1989), respectively. Although this clearly is an exciting start, the future goals are equally clear. The size of the regions involved must be reduced through the identification and analysis of further informative cases and the completion of the chromosome 21 physical map. The genes mapping within each region must be identified, and their potential roles in development be assessed. Finally, but of primary importance, each feature of the phenotype must be defined, at the cellular, physiological, physical, and developmental levels. These studies hold the promise of an ultimate understanding of the molecular basis of the components of the Down syndrome phenotype, including mental retardation, congenital heart disease, immune deficits, risk of leukemia, and the link to Alzheimer disease.

Molecular Structure of Chromosome 21

Knowledge of the physical and genetic maps of chromosome 21 underlies the molecular and cytogenetic methods used to determine DNA sequence copy number in rearranged chromosomes 21. Long-range restriction maps of the long arm of chromosome 21 have recently been constructed using somatic cell hybrids, irradiation reduction hybrids, Southern blot hybridization, and pulsed-field gel electrophoresis (PFGE) (Carritt and Litt 1989; Cox et al. 1990; Gardiner et al. 1990). These approaches have resulted in the placement of over 60 unique DNA markers on the long arm, most of which have been ordered. In addition, a genetic linkage map of the chromosome is emerging (Tanzi et al. 1988; Petersen et al. 1991).

At the International Workshop on Chromosome 21 held on April 2–3, 1990, and attended by representatives of most of the laboratories of the world involved in the mapping of this chromosome, a number of actions relevant to genotype-phenotype mapping of Down syndrome were taken (Cox and Shimizu 1990). A set of 27 ordered, freely available, and well-spaced reference DNA probes spanning the long arm were defined. A set of 12 highly polymorphic genetic anchor markers well spaced along the chromosome were also defined, and a set of five somatic cell hybrids dissecting 21q into well-defined regions were chosen (table 1). These reagents all yield consistent results and are readily available, and it was suggested that kits be prepared for each set of resources. It is now recommended, therefore, that new chromosome 21 arrangements, including translocations, duplications, and deletions, be defined initially in terms of these DNA and genetic markers and hybrids.

Once new chromosome 21 rearrangements are defined in terms of these markers, it will often be possible to refine these boundaries further by using subsets of probes already known to be in the same approximate region as the rearrangement boundary of interest. In general, there is a higher density of well-ordered markers in 21q22→qter, so significantly increased resolution is possible in this region of the chromosome.

Analysis of Data

The workshop participants did not establish a specific approach to the analysis of the clinical, molecular, and cytogenetic data which are expected to be obtained. It is assumed, however, that the data will be handled by methods similar to those that have been used for syndrome identification and classification and for phenotype-karyotype correlations in the past.

There are two principal analytical approaches to the correlation of genotype with phenotype. The first is applicable to features taken separately, not for "patterns," and consists of comparisons of the phenotypes of patients with chromosome duplications, to extract those features that are observed only when specific segments of chromosome 21 are duplicated. The second approach consists of the use of methods of classification to arrange patients with duplications into discrete classes on the basis of their phenotypic relationships and then to look at the chromosomal segments duplicated in such clusters of patients. If clusters so defined are found to correspond to specific duplications, it will be possible to assign a specific phenotype to a chromosome-specific segment.

Several methods of classification developed in the past (Sokal 1974) have been adapted to syndrome definition (Preus and Aymé 1983; Preus et al. 1984).

Table 1

Proposed Chromosome 21 Genetic and Physical Markers

Anchor Hybrids	Reference Markers
	Centromere
ACEM(q11.1)----------	
	D21S16[a]
	D21S13[a,b]
	D21S4[a]
	D21S52[a,b]
4;21(q11.2)----------	----------GM8210
	D21S1/D21S11[b]
	D21S18
1;21(q21.05) ----------	----------GM1881
	D21S8[a,b]
	APP[a]
	D21S111[a]
ACEM(q22.1)----------	
	D21S93
	SOD1[a]
	D21S58[a] ├──*D21S82*[b]
	D21S17[a]
	D21S55[a,b]
10;21(q22.2) ----------	----------GM9542
	├──*HMG14*[a,b]
	D21S3[a]
	D21S15[a,b]
	MX1[a,b]
	D21S19[a] ├──*D21S113*[b]
	CRYA1[a]
	PFKL[a] ├──*D21S112*[b]
	CD18[a]
	COL6A1[a,b]
	S100B
	Telomere

SOURCE.—Modified from Cox and Shimizu (1990).

[a] STS.

[b] Genetic anchor marker.

Experience has shown that *both the analysis of individual features and the classification of patients into homogenous classes (syndromes) by the methods of numerical taxonomy require the development of a list of characters or descriptors showing a high interobserver consistency.* It is to this end that the clinical protocols that follow have been developed. Phenotypic characters may be coded in a binary manner (present/absent), as counterstates (hyperplastic/hypoplastic), or as ordered multistates arranged according to severity or quantitative classification. For the assessment of specific phenotypes related to different duplications, the similarity of each pair of subjects is scored with respect to each character. A similarity matrix based on all possible pattern combinations found in all individuals is constructed, and a total similarity coefficient is calculated for each arrangement of two subjects. This matrix is then analyzed by a combination of clustering or ordination (principal component analysis) techniques to search for identifiable partial duplication syndromes.

The Protocols

The protocols for specifying the cytogenetic and molecular status of each individual and for recording his or her clinical status are presented in the following sections of this report. As much as has been possible, the clinical protocols have been designed so that the

items may be checked off or filled in, and these sections should be largely self-explanatory. However, because of the complexity of the evaluation of central nervous system function, an Appendix describing the rationale for the proposed neurological and psychological evaluations has been prepared. The detailed evaluation of the neurological (including neurophysiological and anatomical) and psychological aspects of chromosome 21 duplications is considered to be of particular importance because of the evidence that particular areas of cognitive functioning and language may be particularly impaired in Down syndrome (see Appendix).

Protocols for the Molecular, Cytogenetic, and Clinical Analysis of Persons with Chromosome 21 Duplications

Molecular Analysis of Chromosome 21 Duplications

The molecular methods for determining DNA sequence copy number are of two types: those based on quantitative densitometry, with or without the analysis of restriction-fragment polymorphisms, and those based on direct estimation of radioactive signals. Both types of methods estimate the copy number of DNA sequences in aneuploid DNAs by comparing the signal from a sequence of unknown copy number with that of a reference sequence the copy number of which in the aneuploid DNA is known. The ratio of these signals in the aneuploid DNA is then compared with the corresponding ratio derived from diploid DNA. All methods are labor intensive and exacting, but they are capable of generating clear results when the appropriate variables are carefully controlled.

Southern blot dosage analysis.—This technique allows the assessment of copy number of any unique chromosome 21 sequence. Southern blots are constructed with restriction enzyme–digested aneuploid and diploid DNAs, hybridized simultaneously with both a probe for a potentially duplicated region of chromosome 21 and a reference probe, and the resulting band signals are measured by densitometry. This process is repeated with a series of chromosome 21 probes until the full extent of the duplicated and nonduplicated regions is defined (see above and table 1).

It is important to note that a variety of technical considerations, including the detailed aspects of gel running, transfer, fixation, and hybridization, all significantly affect the outcome (protocols available from J.R.K. on request). Because of this, criteria for technical acceptance of an autoradiogram should be developed prior to viewing results from a given blot. Reference sequences may be located on the same chromosome (which controls for chromosome loss) or on different chromosomes (which obviates problems with complex rearrangements) and, for best results, should be hybridized simultaneously with the unknown copy-number sequences and should be of similar band size and shape to avoid problems of differential DNA loss between hybridizations. Standardized methods that are known to result in complete enzyme digestions must be used. Multiple lanes of both aneuploid and diploid control DNAs should be run, preferably in an alternating pattern, and the diploid DNAs used for the control should be isolated from the same tissue as the patient sample. Exposures should be carried out to generate bands for analysis that are in the linear range of the film, and statistical analysis should be performed on the results. The standard errors of the resulting ratios determine whether three copies of a given sequence may be differentiated from two copies. It is obviously easier to reach statistical significance for 2:1 copy assessments (as in deletions) than for 3:2 copy assessments (in duplications).

Although exacting, this technique is broadly applicable to the analysis of all DNA sequences and does not require family members to be studied. A further advantage of both this and the method utilizing RFLPs (see below) is that sources of background signal are clearly seen and, in most cases, are separable.

Densitometry.—Densitometric analysis is used to compare radioactive signal intensities obtained by autoradiographic exposures of Southern or slot blots. It is essential that signal intensities are within the linear-response range of both the film and the densitometer being used. The linear range of the film can be determined by exposing the film to radioactivity, X-rays, or white light. In the first case, Whatman 3-mm filter strips can be spotted with ^{32}P-labeled DNA in 1.5-fold increments (Tanzi et al. 1987). In the second method, a U.S. National Bureau of Standards Penetrometer (wedge step gradient) is used in conjunction with X-ray exposure of the film to determine linearity directly in a stepwise fashion. In the third case, the film is flashed with white light at a series of time intervals to determine the linear range. In all of these techniques, the resulting density signals produced on the film are measured after exposure and development, and the values are plotted against the respective exposure modality (amount or duration). The linear range of the film is that in which a constant change in exposure generates a constant change in density when plotted on a log-linear scale. above and below this range,

a given exposure will result in a density change smaller than expected.

In the experimental situation, the absolute density of a band or slot is compared with the linear-range density values determined by the scale constructed. Multiple exposures may be required to bring blot or slot bands to the appropriate range. When weak signals are expected, the film may be preflashed to increase the density uniformly to close to the linear range. Generally, the linear range of most films is short and lies in a limited region of 1–2 OD units. There is a second linear range with a shallow slope in the region below 1 OD. However, it is important to note that different brands and types of film will have different linear-response profiles. Moreover, densitometers, even those of the same brand and model type, also vary in the ranges at which they remain linear.

Analysis of RFLPs.—This method allows the determination of the copy number of polymorphic sequences. The Southern blot method described above may be combined with RFLP analyses to determine DNA sequence copy number. In this case, the existence of three parental alleles is assessed either by the presence of a third distinct band or by the increased copy number of one band relative to a second as judged by densitometry (Tanzi et al. 1987). This technique is the simplest when parental alleles are available for analysis and highly variable multiallelic systems which can distinguish each parental allele have been defined. However, there are few such systems currently available. Therefore, in assessing duplications in which any two of the three alleles present are identical, the analysis requires densitometry and comparison of the ratio of autoradiographic bands in the aneuploid DNA to the normal reference ratio determined for that particular allelic system as seen in the parent of origin. Consequently, the densitometric considerations detailed above still apply, although the analysis is made somewhat easier because the distinction reduces from 3:2 to 2:1. In the absence of parental DNA samples, ratios may be compared with DNAs from normals in the population known to carry the same polymorphism. PCR technology will be particularly useful for this method when sequenced polymorphisms are available for closely spaced alleles. A considerable number of DNA polymorphisms resulting from short sequence repeats are being developed and used in such studies.

Slot blot methods.—Designed as an alternative to the other two methods, the slot blot method also allows the assessment of copy number of any unique chromosome 21 sequence (Blouin et al. 1990). The principle of the method is as follows: DNAs from the person with the chromosome 21 duplication, from a known individual with trisomy 21 (the trisomic control), and from a diploid control are loaded on the same membrane at a series of concentrations by using a slot blotting apparatus. Successive hybridizations with chromosome 21 probes and with a non–chromosome 21 reference probe are then performed, and the resulting signals are quantitated by densitometry. The signals for the reference probe are plotted against those for the chromosome 21 probe for each DNA preparation, and the straight line is calculated by linear-regression analysis. A statistically significant difference between the regression line for the trisomic control and that for the normal subject must be observed. The regression line from the patient with the chromosome 21 duplication is then tested statistically to determine whether it matches with data from either the trisomic control or the normal control (a copy of the gene-dosage program is available from P.M.S. on request). Absence of hybridization background is checked on a slot without DNA and by systematically running control Southern blots. The observation of a significant difference between the trisomic and normal controls suggests that repetitive sequences or other artifacts may not seriously interfere with the estimate of gene copy number. Repeat experiments should give the same results. Attention to using undegraded high-molecular-weight DNA and to complete access of the filter to the DNA (no bubbles) is critical to success. Linearity of signal density must also be considered in this technique.

Direct counting of hybridization signals may be used to evaluate the radioactive signals from both Southern blots and slot blots in all methods described. This has been validated both for the slot blot method utilizing a machine to directly evaluate slot signals (Chettouh et al. 1990) and for methods involving standard scintillation counting (Korenberg et al. 1989). The advantage of this approach is that, although the technical artifacts of Southern blot technology remain, the complexities of densitometry are avoided. However, as yet the methods are not broadly available.

Other potential approaches.—The use of PFGE technology for quantitative Southern blot analysis is not recommended, and its application to the routine definition of chromosome breakpoints is not presently an efficient approach. This is because, despite significant effort, only three breakpoints have been unambiguously detected in over 20 that were examined. Moreover, PFGE is not yet a routine laboratory procedure. Finally, restriction-site polymorphisms, probably due

to differential methylation, may complicate the use of PFGE to search for breakpoints.

An approach which offers some potential advantages for analysis of chromosome 21 rearrangements is the capture of rearranged chromosome 21's in Chinese hamster ovary (CHO) somatic cell hybrids by published procedures (Moore et al. 1977). Use of the Ade-C (*GART*-deficient) CHO mutant has proved almost invariably successful here. These mutants require purines for growth, and the GART gene, which allows growth in the absence of purines, is located in the distal region of band 21q22.1. Molecular probes for the GART gene and flanking regions are now available, so it should be possible to predict with some confidence the outcome of this approach in any given case. Rearrangements involving other chromosomes in translocations often offer additional advantages, since at least one member of the translocation pair can be captured. Selectable markers often exist on the non–chromosome 21 member of the pair, giving additional opportunities to selectively retain rearrangements of interest.

The advantage of capturing a rearranged chromosome in a hybrid is that whether a particular DNA sequence is present becomes an all-or-none result, rather than a 2:1 or 3:2 dosage effect. Disadvantages include the required cytogenetic analysis of hybrids, the rearrangements that occur in human chromosomes maintained in hybrids, the limited number of selective systems, and the requirement for selective media in which to create and maintain hybrids. The choice of whether to construct a hybrid would depend on whether the rearranged chromosome contained the selectable gene and on the resources and skills available to the investigator carrying out the analysis.

Cytogenetic Analysis of Chromosome 21 Duplications

Banding techniques.—Optimum chromosome evaluation of the patient with Down syndrome and other than classic trisomy 21 may require the use of several different cytogenetic techniques and approaches. When a translocation has been identified or when there is an intrachromosomal aberration, high-resolution banding at the 700–850-band level is needed for breakpoint analysis. The method most frequently used to achieve this band level involves synchronization with methotrexate, release of the block, and harvest at a time calculated to yield the highest number of early-metaphase/prophase chromosomes. When even higher band levels are necessary, i.e., to approach the 1,000–2,000-band level illustrated by Yunis (1981), additional procedures are done.

The use of two banding techniques, each giving complementary information, is recommended for breakpoint determination. The overall most crisp and precise technique for banding chromosomes at the 600–850-band level is trypsin G-banding. This technique best reveals the fine gray bands found at high resolution levels in most pale-staining regions, such as at the ends of most chromosomes. Fluorescent R-banding done with the Latt dual stain method, as modified by Schweizer (1980), is the next choice because there are sharper demarcations between bright and dull regions than occur with other fluorescent techniques. Further, it provides R-bands as a contrast to the trypsin G-bands and produces differential brightness among those R-bands that are the G-band-pale regions of many chromosomes. Finally, this technique is singularly effective for the production of high-resolution chromosome bands after in situ hybridization with fluorescent probes described below. Giemsa R-banding after synchronization and BrdU incorporation is also widely used (Viegas-Pequignot and Dutrillaux 1978).

In situ hybridization.—When a subtle chromosome 21 abnormality is suspected but is not confirmed or is unclear with the use of the above banding techniques, in situ hybridization with chromosome 21–specific probes may be used to clarify the chromosomal status. The choice of the particular probe to be used is critical, for, to be informative, the sequence detected by the probe must be within the rearranged segment.

The traditional in situ hybridization technique using tritrium-labeled probes permits the probe sequence to be short (as small as 0.4–0.5 kb), but it requires 5 d or more of exposure in the dark, examination of 50–200 cells, and either construction of an idiogram or statistical analysis for significance. Also, the exact site of hybridization on the chromosome is not evident, because of grain scatter, emulsion thickness, and other factors.

The newer nonradioactive techniques, such as biotinylation of probes which are then detected with fluorescent avidin, generally require a much longer probe sequence (2–3 kb). However, these methods allow precise localization to both chromatids of the chromosomes bearing a homologous sequence. Thus, a more exact localization is possible. Examination of fewer cells is needed for significance, and the process can be completed overnight (Lawrence 1990).

The new in situ hybridization technique of "chromosome painting" may also prove quite useful, especially initially, to identify abnormalities of specifically suspected chromosomes that are not otherwise readily evident. In this technique, a chromosome 21–specific DNA library is amplified, biotinylated, prehybridized with competitor DNA to remove repeated sequences, and then hybridized in a manner similar to that used for unique-sequence probes. If the library is complete, the entire chromosome 21, excluding the regions of highly repeated sequences, will "light up," as will any part of chromosome 21 which may have been translocated to a different chromosome. A similar probe which generates an R-banding pattern may be constructed using PCR technology with primers from the Alu sequence (Baldini and Ward 1991).

Down syndrome with apparently normal karyotype.—Evaluation of the patient with the phenotypic features of Down syndrome but with an apparently normal karyotype requires additional considerations. At least two explanations are possible: undetected mosaicism may be present, or an extremely subtle chromosome 21 translocation/rearrangement may be present.

Mosaicism for standard trisomy 21 in blood may be statistically excluded by the examination of 20 (P = .05) and 100 (P = .01) cells. However, in this situation, a greater number of cells (200–300) is frequently examined. When the test is negative, further analysis of mosaicism in fibroblasts may be warranted by the clinical evidence for Down syndrome.

Undetected chromosome 21 material located elsewhere in the karyotype may be found, as indicated above under "chromosome painting," for relatively large regions. However, smaller regions may be detected with the use of chromosome 21 region-specific DNA sequence mixtures that generate a more intense hybridization signal. Even finer distinction of duplicated regions may be assessed by using either in situ hybridization with well-defined cosmids or gene dosage with unique probes.

Clinical Analysis of Chromosome 21 Duplications

The protocol for recording the phenotype of a person with a chromosome 21 duplication is presented in tabular form suitable for direct reproduction and use. Specific references are provided for the items pertaining to dermatoglyphics and to the nervous system. General reviews containing relevant references for other items in the protocol may be found in the work of Pueschel and Steinberg (1980), Pueschel and Rynders (1982), Epstein (1986), McCoy and Epstein (1987), and Trisomy 21 (Down Syndrome) (1990).

PROTOCOL FOR RECORDING PHENOTYPE OF PERSON WITH CHROMOSOME 21 DUPLICATION

Identifying Information

Date: _____ (month/day/year)

Name: _____ (Last / First)

Date of birth: _____ (month/day/year)

Address: _____

Ethnic group: _____

_____

Place(s) of birth of parents: _____

Telephone number: _____

Pregnancy & Delivery

Was the pregnancy abnormal? Yes _____ No _____ Unknown _____

If yes, was there:

Bleeding or spotting _____
Maternal illness (specify) _____
Maternal skin eruptions _____
Excess weight gain _____
Alcohol _____
Drugs _____
Toxemia _____
Eclampsia _____
Other (specify) _____

Length of gestation _____ weeks
Birth weight _____ kg
Birth length _____ cm
Head circumference at birth _____ cm
Mother's age at birth _____ years
Father's age at birth _____ years

Apgar scores 1 min. _____ 5 min. _____ 10 min. _____

Was the delivery or postnatal course abnormal? Yes _____ No _____ Unknown _____

If yes, was there:

Excessive length of labor (hours) _____
Fetal distress _____
Need for respiratory support _____
Neonatal seizures (specify type, duration, therapy) _____
Sepsis _____
Intracranial hemorrhage _____
Other (specify) _____

Family History

Is there a family history of:

Miscarriage(s)	Yes _____	No _____	Unknown _____	If yes, how many? _____
Abortion(s)	Yes _____	No _____	Unknown _____	If yes, how many? _____
Stillbirth(s)	Yes _____	No _____	Unknown _____	If yes, how many? _____
Birth defects in siblings	Yes _____	No _____	Unknown _____	If yes, how many? _____

If yes, please describe:

Date that possibility of Down syndrome was first suspected. _____ Age: _____
month/day/year

Pedigree:

Photographs of individual at birth and intervals thereafter should be obtained.

Clinical Information

Age of subject: _____years _____months. Does subject have family resemblance? Yes _____ No _____ Unknown _____

Measurements

Height	_____cm	Percentile on DS growth chart (Cronk et al. 1988)	_____%
Weight	_____kg	Internipple distance	_____cm
Head circumference	_____cm	Penis length	_____cm
Inner canthal distance	_____cm	Testicular volume	_____ml
Palpebral fissure length	_____cm	Hand length	_____cm
Ear length	_____cm	Palm length	_____cm
Chest circumference	_____cm	Tanner stage	_____

	Yes	Yes, but parent(s) &/or sib-ling(s) also	Doubtful	No	No, but too young or too old	Unknown	Comments
Head							
Brachycephaly	___	___	___	___	___	___	_____
Open sagittal suture	___	___	___	___	___	___	_____
Third fontanel	___	___	___	___	___	___	_____
Flat face	___	___	___	___	___	___	_____
Other abnormalities/anomalies	___	___	___	___	___	___	_____
Neck							
Broad, short neck	___	___	___	___	___	___	_____
Loose skin of neck							
Webbing	___	___	___	___	___	___	_____
Other abnormalities/anomalies	___	___	___	___	___	___	_____
Eyes							
Upward slant of palpebral fissures	___	___	___	___	___	___	_____
Epicanthal fold(s)	___	___	___	___	___	___	_____
Obstructed lacrimal duct(s)	___	___	___	___	___	___	_____
Blepharitis	___	___	___	___	___	___	_____
Brushfield spots	___	___	___	___	___	___	_____
Peripheral iris hypoplasia	___	___	___	___	___	___	_____
Nystagmus	___	___	___	___	___	___	_____
Strabismus	___	___	___	___	___	___	_____
Congenital cataract	___	___	___	___	___	___	_____
Acquired cataract	___	___	___	___	___	___	_____
Keratoconus	___	___	___	___	___	___	_____
Myopia	___	___	___	___	___	___	_____
Hyperopia	___	___	___	___	___	___	_____
Other abnormalities/anomalies	___	___	___	___	___	___	_____

	Yes	Yes, but parent(s) &/or sib-ling(s) also	Doubtful	No	No, but too young or too old	Unknown	Comments
Nose							
Flat nasal bridge	___	___	___	___	___	___	_____
Anteverted nostrils	___	___	___	___	___	___	_____
Other abnormalities/anomalies	___	___	___	___	___	___	_____
Mouth							
Down-turned corners	___	___	___	___	___	___	_____
Prominent lower lip	___	___	___	___	___	___	_____
Mouth kept open frequently	___	___	___	___	___	___	_____
Protruding tongue	___	___	___	___	___	___	_____
Furrowed tongue	___	___	___	___	___	___	_____
Narrow, short palate	___	___	___	___	___	___	_____
Cleft uvula/palate	___	___	___	___	___	___	_____
Submucous cleft	___	___	___	___	___	___	_____
Other abnormalities/anomalies	___	___	___	___	___	___	_____
Teeth							
Oligodontia	___	___	___	___	___	___	_____
Malformed teeth	___	___	___	___	___	___	_____
Malerupted teeth	___	___	___	___	___	___	_____
Abnormal spacing	___	___	___	___	___	___	_____
Microdontia	___	___	___	___	___	___	_____
Periodontal disease	___	___	___	___	___	___	_____
Other abnormalities/anomalies	___	___	___	___	___	___	_____
Ears							
Prominent upper helix	___	___	___	___	___	___	_____
Adherent lobuli	___	___	___	___	___	___	_____
Helix root crossing concha	___	___	___	___	___	___	_____
Narrow ear canals	___	___	___	___	___	___	_____
Abnormal hearing test (see Neurological)	___	___	___	___	___	___	_____
Other abnormalities/anomalies	___	___	___	___	___	___	_____
Heart							
Atrioventricular canal	___	___	___	___	___	___	_____
Ventricular septal defect	___	___	___	___	___	___	_____
Atrial septal defect	___	___	___	___	___	___	_____
Persistent ductus arteriosus	___	___	___	___	___	___	_____
Mitral valve prolapse	___	___	___	___	___	___	_____
Tetralogy of Fallot	___	___	___	___	___	___	_____
Other abnormalities/anomalies	___	___	___	___	___	___	_____

	Yes	Yes, but parent(s) &/or sib-ling(s) also	Doubtful	No	No, but too young or too old	Unknown	Comments
Behavior disorder	___	___	___	___	___	___	_____
Seizures	___	___	___	___	___	___	_____
Frequent infections	___*	___	___	___	___	___	*Type_____
Leukemia	___	___	___	___	___	___	Frequency_____ _____

Are there other anomalies or abnormalities that have not been mentioned? Yes _____ No _____ Unknown _____

If yes, specify:

Onset of menarche _____years
Onset of menopause _____years
Age of death _____years
Cause of death

Onset of symptoms of Alzheimer disease _____years
Onset of significant intellectual decline _____years

Autopsy findings:

	Yes	Yes, but parent(s) &/or sib-ling(s) also	Doubtful	No	No, but too young or too old	Unknown	Comments
Gastrointestinal Tract							
Tracheo-esophageal fistula							
Duodenal stenosis/atresia							
Annular pancreas							
Imperforate anus							
Hirschsprung disease							
Celiac disease							
Umbilical hernia							
Diastasis recti							
Pyloric stenosis							
Other abnormalities/anomalies							
Musculoskeletal							
Hip dislocation							
Patella subluxation							
Atlantoaxial/atlantooccipital instability							
Hyperextensibility of joints							
Other abnormalities/anomalies							
Skin and Hair							
Alopecia							
Vitiligo							
Keratosis							
Early greying							
Other abnormalities/anomalies							
Hands and Feet							
Brachyclinodactyly							
Single fifth finger crease							
Single transverse palmar crease							
Sydney line							
Increased gap: 1st and 2nd toes							
Webbing between 2nd and 3rd toes							
Other abnormalities/anomalies							
Other							
Hypothyroidism							
Other thyroid disorder							
Diabetes							
Psychiatric disorder							

Laboratory Studies

Hematological

RBC count _____ Hematocrit _____% Differential_____
WBC count _____ Lymphocytes _____% PMNs _____%
Platelet count _____ Size _____
MCV _____ MCH _____ MCHC _____
Fetal hemoglobin _____% Hemoglobin _____g/dl
Erythropoietin (if anemic/plethoric) _____ Cyanotic heart disease Yes _____ No _____
Leukemoid reaction or transient leukemia: Yes _____ No _____ If yes: Age of onset: _____
Highest WBC: _____
Platelet serotonin _____ Age of normalization of WBC:_____

Leukemia: ALL _____ AML _____ AMKL _____ Other _____ M# _____
What was response to therapy? _____ Present Status?_____
Karyotype of leukemia cells: _____
Bone marrow morphology:
Fibrosis: Yes _____ No _____
Megakaryocytic and erythroblastic markers by cytofluorometry:

Immunological

Serum levels: IgG _____ IgA _____ IgM _____ IgG _____
IgG subclasses: _____
CD4 $^+$ _____% CD8$^+$ _____% Ratio _____
TCR-$\gamma\delta^+$ cells _____ CD16 $^+$ NK cells _____
IL-2 production by antigen (bacterial, viral)-stimulated lymphocytes_____ Control (essential) _____

If thymus is available, formalin fixed paraffin blocks should be prepared for morphologic studies
Morphology (Hassall corpuscle size and structure, cortex/medulla - ratio and nature of junction):

[Fresh frozen tissue blocks should be prepared for eventual immunohistochemistry for MHC class II (HLA DR) and ICAM-I expression and in situ hybridization for cytokine gene expression studies.]

Endocrine

TSH _____ FSH _____
T3 _____ LH _____
T4 _____
IGF-1 _____
IGF-2 _____
Thyroid autoantibodies _____
Bone age at <15 years _____yrs at _____yrs

Other

Whole blood serotonin _____
Serum thromboxane level _____
PGE_2 _____

Dermatoglyphic Analysis

Enter pattern codes from table below

		Left	Right
I.	Hallucal area	___	___
II.	Great toe	___	___
III.	Palmar interdigital area-1	___	___
IV.	Palmar interdigital area-2	___	___
V.	Palmar interdigital area-3	___	___
VI.	Palmar interdigital area-4	___	___
VII.	Palmar triradius	___	___

		Left	Right
VIII.	Hypothenar area	___	___
IX.	Simian crease	___	___
X.	Flexion crease on 5th finger	___	___
XI.	Digital pattern-1 (thumb)	___	___
XII.	Digital pattern-2	___	___
XIII.	Digital pattern-3	___	___
XIV.	Digital pattern-4	___	___
XV.	Digital pattern-5	___	___

Hopkins Index (calculated according to Table 3 of Bolling et al. 1971) _____

Pattern codes (Borgaonkar et al. 1971)

I Hallucal area
- 0 - Tibial arch
- 1 - Distal loop - ridge count ≤ 20
- 2 - Distal loop - ridge count > 20
- 3 - Whorl
- 4 - Tibial loop
- 5 - Whorl with seam
- 6 - Tented arch
- 7 - Fibular arch
- 8 - Fibular loop
- 9 - Proximal arch

II Great toe
- 0 - Arch
- 1 - Tibial loop
- 2 - Fibular loop
- 3 - Whorl
- 4 - Tented arch

III-VI Palmar interdigital areas (1 to 4)
- 0 - Absence of pattern
- 1 - Distal loop
- 2 - Whorl
- 3 - Arch
- 4 - Vestigial pattern
- 5 - Whorl with seam
- 6 - Carpal loop

VII Palmar triradius
- 0 - Absence of t
- 1 - t
- 2 - t'
- 3 - t"

VIII Hyothenar area
- 0 - Absence of pattern
- 1 - Ulnar loop
- 2 - Radial loop
- 3 - Carpal loop
- 4 - Whorl
- 5 - Vestigial pattern
- 6 - Distal loop
- 7 - Arch
- 8 - S pattern
- 9 - Tented arch

IX Simian crease
- 0 - Absent
- 1 - Present
- 2 - Doubtful

X Flexion crease 5th finger
- 0 - 2 Creases
- 1 - 1 Crease

XI-XV Digital patterns (digits 1 to 5)
- 0 - Arch
- 1 - Ulnar loop
- 2 - Radial loop
- 3 - Whorl
- 4 - Whorl with seam
- 5 - Tented arch
- 6 - Vestigial loop
- 7 - Vestigial whorl
- 8 - Ulnar loop w/ central pocket
- 9 - Radial loop w/ central pocket

Neurological and Psychological Information

Clinical Neurology

Birth through 4 months

State of infant[a]
Time since last feeding _____hrs.

	Normal/present	Abnormal/absent
Muscle tone	_____	_____
Spontaneous body movements	_____	_____
Blink reflex	_____	_____
Rooting response	_____	_____
Suck	_____	_____
Auditory response	_____	_____
Palmar/plantar grasp	_____	_____
Patellar jerk	_____	_____
Ankle clonus	_____	_____
Plantar responses	_____	_____
Traction response	_____	_____
Ventral suspension	_____	_____
Withdrawal	_____	_____
Automatic stepping	_____	_____
Placing reaction	_____	_____
Moro response	_____	_____

5 months through 6 years

Standard neurological assessment: _____ _____
If abnormal, specify how:

Atlantoaxial/atlantooccipital instability[b] _____ _____

7 years through 18 years

Standard neurological assessment: _____ _____
If abnormal, specify how:

Atlantoaxial/atlantooccipital instability[b] _____ _____

Behavior[c] _____ _____

≥ 19 years

Standard neurological assessment _____ _____
If abnormal, specify how:

Atlantoaxial/atlantooccipital instability[b] _____ _____

Behavior[c] _____ _____
Pre-Alzheimer disease assessment[d] _____ _____

Neuropsychology and Language

Birth through 4 months: no evaluation

5 months through 6 years

Hearing assessment[e] Normal _____ Abnormal: Conductive _____ Sensorineural _____ Mixed _____
Mild _____ Moderate _____ Severe _____

Measures of general cognitive development
For function < 30 months of age level
Bayley Scales of Infant Development[f] Mental age _____ Motor age _____
Ordinal Scales of Psychological Development[g] Level attained _____ Age equivalent _____

For function ≥ 30 months of age level
Kaufman Assessment Battery for Children[h] Mental processing composite age equivalent _____
Peabody Picture Vocabulary Test - Revised[i] Age equivalent _____

Measures of adaptive behavior
Vineland Adaptive Behavior Scale[j] Composite age equiv. _____ Communication age equiv. _____ Motor age equiv. ____

Language structure
Mean length of utterances[k] MLU _____ Age equivalent _____
Reynell Developmental Language Scales[l] Expressive age equiv. _____ Comprehension age equiv. _____
Temple University Short Syntax Inventory[m] Age equivalent _____

7 years through 18 years

Measures of general cognitive development
Kaufman Assessment Battery for Children[h] Mental processing composite age equivalent _____
Peabody Picture Vocabulary Test - Revised[i] Age equivalent _____
Vineland Adaptive Behavior Scale[j] Composite age equiv. _____ Communication age equiv. _____ Motor age equiv. ____
Wechsler Intelligence Scale for Children - Revised[n] _____

Language structure
Mean length of utterances[k] MLU _____ Age equivalent _____
Patterned Elicitation Syntax Test[p] Age equivalent _____
Test for Auditory Comprehension of Language - Revised[q] Age equivalent _____
Test of Language Development[o] _____

Phonology

Arizona Test for Articulation[s] — Age equivalent _____ — % intelligibility _____

Shriberg/Kwiatkowski intelligibility measure[t] — % consonants correct _____ — % intelligibility _____

Memory function[u]

Verbal memory — Word span _____ — Age equivalent _____

Non-verbal memory — Object span _____ — Age equivalent _____

≥ 19 years

All tests recommended for 7 through 18 years

Assessment for dementia[v] _____

Neurophysiology

All Ages

Auditory Brain Stem Evoked Responses[w]

Central condition times at 60 dBHL (in z scores)

Latency wave I	_____
Latency wave V	_____
I-V interval	_____
I-III interval	_____
III-V interval	_____
I-II interval	_____
III-IV interval	_____
IV-V interval	_____

Latency-Intensity & Amplitude-Intensity Functions (in z scores)

	Wave V	
	Latency	Amplitude
dBHL 0	_____	_____
5	_____	_____
10	_____	_____
15	_____	_____
20	_____	_____
40	_____	_____
60	_____	_____
80	_____	_____

Latency of wave V at 20 dBHL minus that at 60 dBHL (in z scores) _____

Click Threshold: weakest level at which wave V is reliably present (in dBHL) _____

≤7 years

Event Related Potentials[x] (in z scores)

		Novel Stimuli		
		Nc	P3a	
Latency:	auditory	_____	_____	N_C at F_z
	visual	_____	_____	P3a at C_z
Amplitude:	auditory	_____	_____	N_C at F_z
	visual	_____	_____	P3a at C_z
Scalp topography:	auditory	_____	_____	F_z/P_z ratio
	visual	_____	_____	

≥7 years

Event Related Potentials[y] (in z scores)

		Target Stimuli		Novel Stimuli		
		Nc	P3b	Nc	P3a	
Latency:	auditory	_____	_____	_____	_____	N_C at F_z
	visual	_____	_____	_____	_____	P3a at C_z
						P3b at Pz
Amplitude:	auditory	_____	_____	_____	_____	N_C at F_z
	visual	_____	_____	_____	_____	P3a at C_z
						P3b at Pz
Scalp topography:	auditory	_____	_____	_____	_____	F_z/P_z ratio
	visual	_____	_____	_____	_____	

Neuroimaging (Repeat all tests yearly once dementia is suspected in persons >40 years old)

All ages

Quantitative MRI scan
- Lobar and gyral dimensions _____
- Ventricular volumes _____
- CSF volume _____
- Whole brain volume _____

≥ 19 years

PET scan ("resting state" glucose metabolism)[z] _____

Neuropathological Examination

All ages

Brain weight	_____ g				
Frontal-occipital diameter	_____ cm			Biparietal diameter	_____ cm
Malformation of superior temporal gyrus	Yes	_____	No	_____	
Size in relation to cerebral hemisphere (ratio)	Cerebellum	_____	Brainstem	_____	

Microscopic

Is there a decrease in number of small neurons in cerebral cortex (by quantitative comparison with control)? Yes _____ No _____

≥ 19 years

Neurofibrillary tangles and senile plaques[aa]	Yes	_____	No	_____
Premature age-related myelin pallor[bb]	Yes	_____	No	_____
Ventricular dilatation	Yes	_____	No	_____

a Prechtl (1977)

b Evaluation for the presence of atlanto-axial dislocation: History - neck/back pain, difficulty walking, weakness in arms, acquired urinary incontinence. Examination findings - limitation of neck motion, weakness in arms/legs, diminished biceps, triceps, brachioradialis reflexes, increased tendon jerks at knee and ankle, clonus, plantar extensor responses, sensory level. Laboratory studies - radiographs of neck in extension/flexion, possible CT or MRI of cervical spine (if clinically indicated), somatosensory evoked responses in the legs (if clinically indicated).

c Screening test for neurobehavioral disorders including psychosis, compulsive behaviors, stereotyped mannerisms, self injurious behaviors, and attention deficit disorder (see Behavioral Checklist, Achenbach and Edelbrock 1983).

d Pre-Alzheimer disease assessment: History - change in personality (loss of motivation, increased irritability), decline in activities of daily living skills, increasing awkwardness of gait, onset of a new seizure disorder (type/length/medication), urinary/fecal incontinence.

e To be classified as normal hearing, most studies require a cutoff of 25 dBHL ISO for 500, 1000, 2000, 4000, and 6000 Hz bilaterally. All speech and language testing should be carried out by a speech pathologist.

f Bayley (1969)

g Uzgiris and Hunt (1975)

h Kaufman and Kaufman (1983)

i Dunn and Dunn (1981)

j Interview edition, survey form (Sparrow et al. 1984)

k Brown (1973). Age norms and details on coding are provided in Miller (1981)

l Reynell (1977)

m Gerber and Goehl (1984)

n For child ≥ 12.5 years approaching normal intelligence (Wechsler 1974)

o Newcomer and Hammill (1988) - when appropriate in individuals with near normal language

p Young and Perachio (1983)

q Carrow-Woolfolk (1985)

r The Illinois Test of Psycholinguistic Abilities (Kirk et al. 1968). Two subtests should be administered to children 7 years and above. The express reason for including this test is to replicate the robust finding of relatively advanced motor expression in DS children compared to overall performance and compared to other mentally retarded individuals matched on age and IQ (Bilovsky and Share, 1965; McCarthy, 1965). The subtests to be included are "Grammatic Closure" (a test of the ability to complete sentences with words that have the correct syntactic markers) and "Manual Expression" (the ability to express concepts with manual gestures).

s Fudala and Reynolds (1986)

t Shriberg and Kwiatkowski (1982)

u Verbal memory subtests from the Kaufman battery including digit span and memory for objects. This should be supplemented with a word span measure following Varnhagen et al. (1987). Nonverbal memory subtests from the Kaufman battery involving memory for handshape. This shoud be supplemented by a block tapping span from Corsi (1972).

v Neuropsychological battery from Haxby (1989) designed to assess dementia in Down syndrome: tests of praxis, the ability to create longterm memories, recall of personal information and temporal orientation, visuo-spatial construction, and visuoperceptual discrimination.

w Widen et al. (1987)

x Event related potential (ERP) testing to novel stimuli: visual and auditory analogs of Karrer and Ackles (1988).

y From 7 years on, use ERP testing to target and novel stimuli: Courchesne et al. (1977); Lincoln et al. (1985). Subjects press a button in response to target stimuli only.

z Baseline PET scan should be considered between 19 and 35 years. Repeat PET scan should be considered at > 35 years if there are findings of dementia.

aa This requires samples from hippocampus, amygdala, and association cortices with special staining for senile plaques and neurofibrillary tangles.

bb Premature age-related myelin pallor is best seen in the occipital pole - particularly in the corona radiata.

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Appendix

Development of Protocols for Neurological and Psychological Evaluations of Persons with Down Syndrome

The clinical neuroscience of Down syndrome may be best viewed as a series of developmental sequences, the manifestations of which are age dependent. For this reason, the clinical protocols have been organized into epochs which have special significance for the development and senescence of the individual with Down syndrome. The following age categories have been observed in protocol development:

I. Birth–4 mo
II. 5 mo–6 years
III. 7–18 years
IV. 19–35 years
V. Greater than 35 years

Although any such partition of development is arbitrary, these categories mark many of the neurological milestones seen in Down syndrome. In the neonatal period (I), the presenting neurological reflexes are characteristic of the disorder. In infancy and early childhood (II), language development seems to present specific problems for the child with Down syndrome, and there is a deceleration in the rate of head-circumference growth. During the midchildhood years (III), there are further consolidations of language and cognitive development, and the clinical problem of atlantoaxial/atlantooccipital instability may appear. In the remaining epochs (IV and V), the neurobiological observations pertaining to accelerated loss of function and to Alzheimer disease require a specific series of measurements and observations.

The purpose of the clinical neuroscience data base is to acquire phenotypic information about the nervous system of the individual with Down syndrome, information which may ultimately correlate both with clinical measures in other systems and with genotypic finding. Because of the complexity of many of these measures, we have chosen to categorize them according to the following disciplines: clinical neurology, neuropsychology and language, neurophysiology, neuroimaging, and neuropathology.

Clinical Neurology

The developing nervous system has a limited number of recognizable clinical manifestations of disease, and these are often not specific for etiology. For this reason, there is no pathognomonic neurological presentation for Down syndrome, although several features occur with regularity. One of these is generalized hypotonia, which is present at birth and persists throughout early childhood. The etiology of this muscular hypotonia remains unknown. In addition to hypotonia, Cowie (1970) has noted delayed dissolution of early reflexes and automatisms—specifically, grasp reflexes, Moro response, and automatic stepping. Abnormal or deficient responses have included the traction response, position in ventral suspension, and patellar jerk. These findings are reported to correlate well with developmental performance at age 10 mo. A consequence of ligamentous laxity is an atlantoaxial/atlantooccipital instability (Pueschel et al. 1987) which poses risk to the cervical spine of perhaps 15%–20% of children with Down syndrome (Pueschel and Scola 1987), although symptomatic manifestations are reported to be rare.

As the child with Down syndrome matures to an adult, another series of neurological concerns arise in regard to Alzheimer disease and precocious aging (Lott 1982). First described by Jervis (1948), it has been established that virtually all brains from individuals with Down syndrome over age 40 years show the neuropathological features of Alzheimer disease but that a considerably smaller percentage actually develop clinical symptoms of the disorder (Oliver and Holland 1986). When symptomatic, individuals with Down syndrome and Alzheimer disease appear to show a degenerative syndrome beginning with personality change, loss of daily living skills, and apraxias with progression to a neurovegetative state over months to years. The clinical problem of Alzheimer

disease in Down syndrome has received much attention and has been approached from each of the disciplines in the protocol.

Neuropsychology and Language

Over and above the more cognitive impairments, specific deficits in linguistic structure (both syntax and phonology) and in auditory verbal memory occur in Down syndrome (Burack et al. 1988; Fowler 1988, 1990). Unfortunately, however, these areas are not typically represented on omnibus IQ tests. The battery of measures outlined in the protocol includes a general assessment of cognitive functioning at each age, as well as the means to construct characteristic patterns. The goal with each subject is to determine whether there is a disparity between (*a*) the mental age functioning on general IQ measures and (*b*) age-equivalent scores on syntax, phonology, or memory. To assure reliable assessment of individual constructs (IQ, memory, syntax, phonology), three separate measures are recommended for each. The suggested measures were selected as being especially analytic, as having valid age norms, and for clarity and availability. For languages other than English, speech pathologists should select measures which assess the areas of interest in a similarly analytic fashion. In addition, it is critical to assess hearing (both pure tone and impedance), structural defects affecting phonology (e.g., cleft palate), and visual acuity, before interpreting the results of language and cognitive testing. A questionnaire is helpful in documenting variables which may influence cognitive and language measures—e.g., variables such as history of chronic ear infections, participation in early-intervention programs, parental education levels, and time spent in institutionalized settings. In general, the use of multiple measures allows for convergence of findings, which tends to make the results more meaningful than does a single measure alone.

The *measures of general cognitive assessment* will provide an IQ score, a level of cognitive functioning, and a mental age equivalent. The latter variable is the most helpful, since it can be compared with performance on specific measures of linguistic structure, intelligibility, and memory. When justified by the child's level of functioning, the examiner should move up to the next level of testing. For each age epoch, three measures of general cognitive functioning are recommended:

1. The first measure is a well-normed omnibus measure of general intelligence which incorporates motor, performance, and verbal factors and which is relatively free of acquired knowledge. The *Bayley Scales of Infant Development* (Bayley 1969) is recommended for the youngest children and has been widely used in Down syndrome research. For children functioning at the preschool level and beyond, the *Kaufman Assessment Battery for Children* (K-ABC; Kaufman and Kaufman 1983) is well standardized for both normal and exceptional children. The *Wecshler Intelligence Scales for Children—Revised* (Wechsler 1974) should be utilized only with higher-functioning older individuals.

2. As a second measure of general intelligence, the battery includes a separate measure dedicated to receptive vocabulary, often used as the core variable in general intelligence measures such as the Stanford-Binet. The Peabody Picture Vocabulary Test—Revised (PPVT-R; Dunn and Dunn 1981) serves this purpose well; the PPVT-R has been standardized on the normally developing 30 mo–adult population.

3. The third measure recommended is the *Vineland Adaptive Behavior Scales: Interview Edition, Survey Form* (Sparrow et al. 1984). This measure of adaptive functioning is appropriate from infancy to adulthood and has been extensively used in individuals with Down syndrome.

These measures provide verbal and nonverbal subscores, as well as scores on subtests specific to memory. In Down syndrome, the findings show that measures of IQ, general cognitive functioning, and mental age are at least 2 SD below the norm and that performance on these global anchor measures exceeds performance on measures of language, phonology, and memory. All three tests provide norms for both English- and Spanish-speaking children; the PPVT-R and Vineland tests could be adapted to other languages, allowing comparison of absolute scores.

In individuals with Down syndrome, the development of *linguistic structure* is impaired not only relative to general cognitive development but also relative to other "verbal" abilities, including communicative function (Beeghly et al. 1990) and vocabulary knowledge (Evans 1977; Miller 1988). This deficit in language structure is apparent in the length and complexity of sentences generated spontaneously, as well as in the structure of sentences elicited under more controlled conditions. In the early stages of language development, a particularly useful index of overall language level is the *mean length of utterances* (MLU) produced by the child (Brown 1973; Miller 1981). This measure is widely used with normally developing children and in special populations as well (Scarbor-

ough et al., in press). It is important to complement an MLU measure with an elicitation task and with a measure of sentence comprehension. In children functioning below the normal 3-year-old's language level, the *Reynell Developmental Language Scales* (1969) assess comprehension and some elicited production; the *Temple University Short Syntax Inventory* (1984) provides a brief check on sentence-imitation skills. At more advanced language levels, these needs can best be met with the *The Patterned Elicitation Syntax Test* (1983) and the *Test of Auditory Comprehension of Language–Revised* (Carrow-Woolfolk 1985). The latter test is relatively free of a lexical confound and has proved sensitive to individual differences in language function in Down syndrome (Sommers and Starkey 1977).

Phonological development is a notable area of difficulty in Down syndrome (Crosley and Dowling 1989). A standardized articulation test, the *Arizona Test of Articulation* (Fudala and Reynolds 1986), measures the adequacy of the subject's production of segments in isolated contexts and can be scored for language age and percent of intelligibility. To complement this, we recommend a measure of intelligibility of running speech (Shriberg and Kwiatkowski 1982; Shriberg 1986).

Auditory verbal memory has long been noted as a major psycholinguistic deficit in Down syndrome (Varnhagen et al. 1987). This deficit is maintained for both visual and verbally presented material which must be held in verbal store. In addition, memory is of some interest because older demented individuals with Down syndrome have diminished abilities to form new long-term memories, compared with individuals with Down syndrome of the same age who are not demented (Haxby 1989). To closely examine the process of cognitive change in older individuals with Down syndrome, we recommend the battery used by Haxby for groups IV and V.

Neurophysiological Measures

Neurophysiological testing using event- related potential (ERP) techniques should be performed on all age groups. Testing will fall into two domains: recordings of auditory brain stem–evoked responses (ABERs) and recordings of auditory and visual ERPs associated with novelty and attention. These testing domains have been of particular interest in studies of Down syndrome.

In regard to ABER assessment, neural responses I–V will be recorded (Wilden et al. 1987). The interpeak intervals of responses I-II and III-IV have been noted to be relatively reduced, and the interpeak interval of IV-V has been noted to be prolonged (Squires et al. 1986). The latency-intensity function of wave V is typically steeper than normal in subjects with Down syndrome, especially in those with high-frequency (8,000 Hz) hearing loss (Squires et al. 1986; Widen et al. 1987), and wave V response amplitude is also reduced (Widen et al. 1987).

Novelty-and-attention-related ERP responses (e.g., P3b, Nc, P3A) will be obtained as per Lincoln et al. (1985) and Courchesne (1977) for Down syndrome age groups III–V and in a fashion analogous to Karrer and Ackles (1988) for Down syndrome age groups I and II. Auditory ERP responses will be elicited by target phonemes and novel sounds; the visual ERP protocol will be analogous to the auditory procedure. For all novelty-and-attention ERP responses (e.g., P3a and P3b), peak *latencies* are longer than normal in subjects with Down syndrome (Lincoln et al. 1985; Muir et al. 1988). Also, ERP response *amplitudes* (e.g., P3b) tend to be smaller than normal in Down syndrome age groups III–V (Lincoln et al. 1985; Muir et al. 1988). In addition, the *scalp topography* of the attention-related P3b response is abnormal in Down syndrome (Lincoln et al. 1985). These abnormalities may be more exaggerated in aged and demented subjects with Down syndrome (Muir et al. 1988).

Neuroimaging

The neuroimaging protocols follow in part from the neuropathological data concerning brains of persons with Down syndrome (see below) and from quantitative computed tomography (CT) studies of adults with Down syndrome with or without dementia. The most accessible neuroimaging procedures which are available for children with Down syndrome and which correlate with other protocol data are CT and magnetic resonance imaging (MRI) brain scans. Volumetric MRIs should be carried out periodically to assess deceleration of development, cerebellar growth, and the appearance of the superior temporal gyrus. When quantitative myelin studies are feasible through MRI, they may be of additional use.

Young adults (18–35 years of age) with Down syndrome have brain volumes which, when assessed quantitatively with CT, are smaller than those in age-matched controls. However, when brain volumes are normalized to body height (persons with Down syndrome are shorter than controls), there is no significant difference between subjects with Down syndrome

and controls (Schapiro et al. 1987). Thus, there is no in vivo imaging evidence of brain-growth reduction that is disproportionate to short stature in young adults with Down syndrome.

In older adults (more than 45 years of age) with Down syndrome who are demented, quantitative CT studies have demonstrated both increased cerebrospinal fluid (CSF) volume in cross-sectional studies and accelerated rates of dilatation of the lateral ventricles in longitudinal studies, as compared with age-matched controls and with older nondemented adults with Down syndrome (Schapiro et al. 1989*b*). The argument that neuroimaging analysis may correlate strongly with genotype is supported by the observation of Schapiro et al. (1989*a*) that an individual with mosaic Down syndrome, with three copies of chromosome 21q, was not mentally retarded but at age 45 years developed dementia, with an enlarged right lateral ventricular volume (as compared with mean ventricular volume in nondemented older individuals with Down syndrome) and with reduced metabolism in parietal association cortical areas (see below). Correlation of such findings with the neuropsychological battery of Haxby (1989) seems particularly promising. In the future, MRI will be preferred to CT for assessing volumes of intracranial structures in Down syndrome, as MRI provides better measures of gyral and lobar dimensions, being free of the CT bone-hardening artifact. CT, in which cross-sectional areas in the CT slices are summed by taking into account slice thickness and interslice separation, remains adequate for assessing lateral ventricular and whole-brain volumes (Schapiro et al. 1987, 1989*b*).

Data regarding positron emission tomography (PET) scanning in Down syndrome are new, and this procedure is not widely available at present. In young adults with Down syndrome who are not demented, there is a subtle abnormality in the correlations between "resting state" glucose metabolic rates in Broca's speech area and in frontal/parietal cortical regions (Horwitz et al. 1990), but absolute glucose metabolic rates do not differ from values in age-matched controls (Horwitz et al. 1990). In older demented adults with Down syndrome, glucose metabolic rates are reduced in parietal and temporal motor areas. These reductions are not found in young (18–35 years of age) or older (more than 35 years of age) nondemented adults with Down syndrome but are identical to PET-metabolic reductions in patients with Alzheimer disease in the population with Down syndrome (Schapiro et al. 1988).

Neuropathology

An understanding of the significance of the structural data concerning the development of the brain in Down syndrome ultimately depends on neuropathological observations. The following features of Down syndrome neuropathology are characteristic: (1) foreshortening of the frontal/occipital diameter of the forebrain (Zellweger 1977), (2) narrowing of the superior temporal gyrus (Kemper 1988), (3) disproportionately small cerebellum and brain stem (Crome et al. 1966), and (4) normal or nearly normal brain weight and head circumference at birth, with slowed postnatal growth rate in both of these parameters (Roche 1966; Benda 1971). Quantitative MRI can document the first three characteristics at any age, and the sequential pathology of characteristic 4 is best seen with serial MRI at birth–6 years. A characteristic feature of the cerebral cortex is a decrease in the small neurons in all cortical layers (Ross et al. 1984). This can be seen only at autopsy study and requires age-matched control material taken from the same areas as the samples taken from the Down syndrome brain, with processing in an identical manner.

An underlying assumption is that age-related atrophic changes in the brain occur prematurely in Down syndrome (Zellweger 1977; Kemper 1988), particularly in subjects who begin to demonstrate dementia (Schapiro et al. 1988, 1989*b*). These changes include mineralization of the globus pallidus, dilatation of the lateral and third ventricles, and the accumulation of senile plaques and neurofibrillary tangles in the cerebral cortex, hippocampus, and the amygdala. The dilatation of the ventricles and mineralization of the basal ganglia can be documented during life by MRI or CT scans in those who are more than 35 years old. The demonstration of senile plaques and neurofibrillary tangles depends on the use of special stains in selected brain regions at the time of autopsy and occurs in subjects older than 35 years. Their presence in large numbers is considered to be evident for Alzheimer disease in individuals with Down syndrome (Wisniewski et al. 1985).

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Down Syndrome: Molecular Mapping of the Congenital Heart Disease and Duodenal Stenosis

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Summary

Down syndrome (DS) is a major cause of congenital heart and gut disease and mental retardation. DS individuals also have characteristic facies, hands, and dermatoglyphics, in addition to abnormalities of the immune system, an increased risk of leukemia, and an Alzheimer-like dementia. Although their molecular basis is unknown, recent work on patients with DS and partial duplications of chromosome 21 has suggested small chromosomal regions located in band q22 that are likely to contain the genes for some of these features. We now extend these analyses to define molecular markers for the congenital heart disease, the duodenal stenosis, and an "overlap" region for the facial and some of the skeletal features. We report the clinical, cytogenetic, and molecular analysis of two patients. The first is DUP21JS, who carries both a partial duplication of chromosome 21, including the region 21q21.1-q22.13, or proximal q22.2, and DS features including duodenal stenosis. Using quantitative Southern blot dosage analysis and 15 DNA sequences unique to chromosome 21, we have defined the molecular extent of the duplication. This includes the region defined by DNA sequences for *APP* (amyloid precursor protein), *SOD1* (CuZn superoxide dismutase), *D21S47, SF57, D21S17, D21S55, D21S3,* and *D21S15* and excludes the regions defined by DNA sequences for *D21S16, D21S46, D21S1, D21S19, BCE I* (breast cancer estrogen-inducible gene), *D21S39,* and *D21S44.* Using similar techniques, we have also defined the region duplicated in the second case occurring in a family carrying a translocation associated with DS and congenital heart disease. This region includes DNA sequences for *D21S55* and *D21S3* and excludes DNA sequences for *D21S47* and *D21S17.* The DS molecular-overlap region is defined by the three DNA sequences duplicated in both patients and includes *D21S55, D21S3,* and *D21S15.* These studies provide the molecular basis for the construction of a DS phenotypic map and focus the search for genes responsible for the physical features, congenital heart disease, and duodenal stenosis of DS.

Introduction

Down syndrome (DS) is a major cause of mental retardation and congenital heart disease, affecting over 200,000 persons in the United States (reviewed in Epstein 1986). In addition to these, other major features include a characteristic facies, skeletal anomalies, abnormalities of the immune system, an increased risk of leukemia, and an Alzheimer-like presenile dementia. Although usually caused by trisomy 21, a subset of these features, including the classical facies and mental retardation, may be caused by duplication of band q22 (Niebuhr 1974). Subsequent work based on cytogenetic analyses assigned the likely chromosomal location of these features to proximal band 21q22 (Poissonnier et al. 1976). More recent work based on molecular analyses has suggested that the facial and some of the other physical features may be associated with a duplication of the chromosomal region that includes the DNA sequence *D21S55* (Rahmani et al. 1989). Our recent work analyzing a family with DS and a chromosome 21 translocation has defined molecular markers for and has narrowed the cytogenetic region likely to contain the genes responsible for DS

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features—including the congenital heart disease, facial features, some of the hand and foot abnormalities, and a part of the dermatoglyphic features—to the region of distal band 21q22.1-q22.3 (Korenberg et al. 1990). We now present further molecular analyses of DS individual DUP21NA (patient IV-1) from this family and of another DS individual, DUP21JS, with duodenal stenosis (DST). The results define a small molecular-overlap region for the DS facial, dermatoglyphic, and other physical features and, in addition, define molecular markers for chromosomal regions that may contain the genes for the congenital heart disease and congenital DST of DS.

Material and Methods

Molecular Analysis

The DNA sequence probes used in these investigations are all present in single copy in the human genome, unique to chromosome 21. FB68L is a cDNA probe corresponding to the 3′ end of the APP locus (Tanzi et al. 1987). The cDNA probe for SOD1 was a gift from Y. Groner (Sherman et al. 1983). BCEI, a breast cancer estrogen-induced mRNA, was from ATCC (Moisan et al. 1985). *D21S1* (228C), *D21S3* (231C), and *D21S55* (518–8B), from P. Watkins, are DNA sequences subcloned in either pBR328 (200 series) or pBR322 (500 series), as described by Watkins et al. 1985*a*, 1985*b*). Probes for *D21S39, D21S44, D21S46, D21S47,* and SF57 are single-copy DNA sequences (Korenberg et al. 1987). The genomic clones for *D21S16* (pGSE9), *D21S17* (pGSH8), *D21S15* (pGSE8), and *D21S19* (B3) are from G. Stewart (Stewart et al. 1985*a*, 1985*b*). The chromosome 17 single-copy probe, HHH202 (D17S33) (Nakamura et al. 1987), was used as the reference for all experiments. Approximate map positions for these are indicated in figure 5, at the right of the ideogram. Order of sequences is as indicated by physical mapping studies (Korenberg et al. 1989; Gardiner et al. 1990; Owen et al. 1990; T. Falik-Borenstein and J. R. Korenberg, unpublished data). Brackets indicate groups of probes for which order has not been established.

Procedures for DNA isolation and digestion, agarose gel construction, Southern blotting, probe labeling, hybridization, and autoradiogram development were conducted as described by Korenberg et al. (1989). All probes were isolated as DNA fragments. DNAs for DUP21JS were obtained from a fibroblast cell line, for DUP21NA from a lymphoblastoid cell line, and for the diploid control, from peripheral blood. Approximately 3–4 μg of DNA digested with *Eco*RI (Bethesda Research Laboratories) were run per lane, in 1.2% agarose gels. As previously described, at least two independent gels were run, each with 16 lanes of alternating DNAs from patient and control. Probes were hybridized simultaneously in groups of three to five, including the chromosome 17 reference probe for each Southern blot membrane produced.

Cytogenetic Analysis

Extended chromosome preparations were made from peripheral blood lymphocyte cultures by using methotrexate synchronization (Yunis 1976). Metaphase-chromosome preparations were made from skin-fibroblast cultures by using standard procedures. The chromosomes were stained by G-banding (GTG) and R-banding (RHG).

Calculation of DNA Sequence Copy Number

DNA sequence copy number in DS patient DUP-21JS was determined from ratios representing the mean of 8–13 paired measurements of autoradiographic band densities obtained with DNAs from DUP21JS and diploid control. Autoradiograms analyzed were those generated by exposure of Kodak XAR film for the time required to bring the band signals to an approximately linear range of the film (determined using a National Bureau of Standards penetrometer). Each autoradiographic band was measured by a Helena EDC densitometer, and the area under the curve was integrated by computer. Individual paired measurements were calculated as the ratio of the chromosome 21 test sequence to the chromosome 17 reference sequence (*D17S33*) in DNA from DUP21JS, versus this same ratio in the control DNA. For DUP21JS, ratios of ~1.5 indicate three copies of the test DNA sequence versus two copies of the reference sequence, whereas ratios of ~1.0 indicate two copies of both the test DNA sequence and the reference DNA sequence. The mean ratios were shown by *t*-test to be significantly different from 1.0 ($P < .001$) or 1.5 ($P < .01$; $P < .001$). Ninety-five-percent confidence intervals (95% CI) are shown. Similar techniques were used for the analysis of DUP21NA.

Results

Phenotype and Chromosomal Analysis

Patient DUP21JS.—This individual, the product of an uncomplicated 41-wk gestation, was born to a 25-

Table 1

Clinical Features of DS Patient DUP21JS and of Four Affected Individuals

	DUP21JS[a]	DS Family[b] (total no./no. tested)
Age at evaluation (years)	2.0	.5, 2, 6.5, 34
Microcephaly	(−3 SD)	0/2
Brachycephaly		
Flat occiput	+	0/2
Hypotonia	+	1/3
Lax ligaments	+	2/2
Poor suck at birth		2/2
Delayed milestones	Motor speech	3/3
Short stature	(−3 SD)	0/2
Failure to thrive		1/2
Dementia	−	0/3
Flat facies	+	4/4
Up-slanted palpebral fissures	+	4/4
Epicanthic folds	+	4/4
Telecanthus	+	2/2
Brushfield spots	−	
Flat nasal bridge	+	4/4
Dentition abnormal	−	1/1
Macroglossia	−	2/2
High palate	−	0/2
Open mouth	+	2/2
Ears cupped or low set	−	0/2
Ears small	−	0/2
Short neck	−	0/2
Heart anomaly	PDA	2/4
Gut anomaly	DST	0/4
Other (exstrophy of bladder)	+	0/4
Broad hands	+	2/4
Brachydactyly	Mild	
Fifth-finger clinodactyly	+	2/4
Wide space between first and second toes	+	
Finger pads		1/2
Third interdigital loops		2/2
Hypothenar patterns		2/3
Distal axial triradius (t″)		2/2
Single transverse palmar crease		1/2
Hallucal fibular loop		2/2
First interdigital loop		2/2
Sole open-field patterns		2/2

NOTE.—A plus sign (+) indicates presence of feature; a minus sign (−) indicates absence of feature; and a blank space indicates that no information was available.

[a] PDA = patent ductus arteriosus, closed spontaneously; and DST = duodenal stenosis.

[b] Data, for the DS patients from a single DS family, are adapted from Korenberg et al. (1990).

year-old G2P2 healthy mother and unrelated father. Family history was negative, and mother was adopted. Birth weight was 3 kg (−1.0 SD), length was 47.8 cm (1.7 SD), and head circumference was 33 cm (−1.5 SD). The following were noted at birth (table 1): DS features were exstrophy of the bladder and DST, both of which were surgically repaired. Laboratory results included a hematocrit of 60 on day 1 and of 52 on day 5 with a platelet count of 82,000 and a white-cell count of 17,400 (53% polymorphonucleocytes [PMN], 18% band PMNs, 25% lymphocytes, 2% monocytes, and 2% basophils). Cranial ultrasound on day 1 revealed mildly prominent lateral ventricles. There was a small patent ductus arteriosus that closed spontaneously

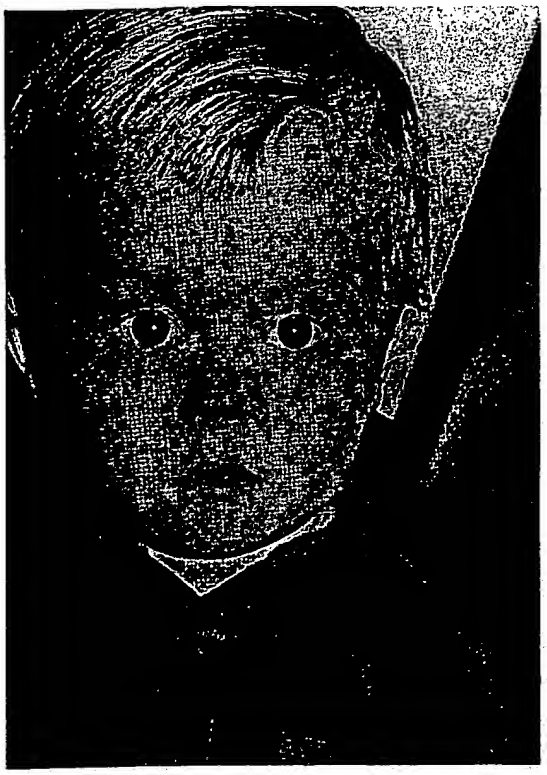

Figure 1 Photograph of DS patient DUP21JS at age 2 years.

and a normal echocardiogram. There have been no problems with infections other than occasional otitis media and urinary-tract infections secondary to his surgeries. Growth has continued since the age of about 6 mo, at below the 3% for height, weight, and head circumference. Motoric developmental delay was manifested by onset of cruising at 18 mo and walking at 23 mo. Speech was delayed, and, when the patient was 2 years of age, his vocabulary was about 12 words. No formal testing was done, and the family was not available for further evaluation. The results of the physical exam showing the features of DS at age 2 years are shown in figure 1 and table 1.

Figures 2 and 3 illustrate the results of cytogenetic analysis of DUP21JS. A total of 45 cells from the blood lymphocyte cultures and 10 cells from the skin fibroblast cultures were found to have an abnormal chromosome 21 with partial duplication of the long arm. By cytogenetic analysis, the diagnosis appears to be 46,XY,inv dups ins (21) (q22.11q22.2q21.1). This results in a duplication of the region beginning in mid 21q21.1 and extending to mid q22.2. However, we cannot rule out that the duplication is part of a more complex chromosome rearrangement. Parental chromosomes were normal.

Patient DUP21NA.—This individual and three other members of her family exhibit the features of DS listed in table 1 and described in detail by Korenberg et al. (1990). These include the facial and hand features, in addition to a part of the mental retardation and the congenital heart disease of DS. These DS individuals carry an unbalanced translocation of chromosome 21, resulting in a duplication of either 21q22.1 distal or q22.2 through qter, defined by both cytogenetic and preliminary molecular analyses (Korenberg et al. 1990).

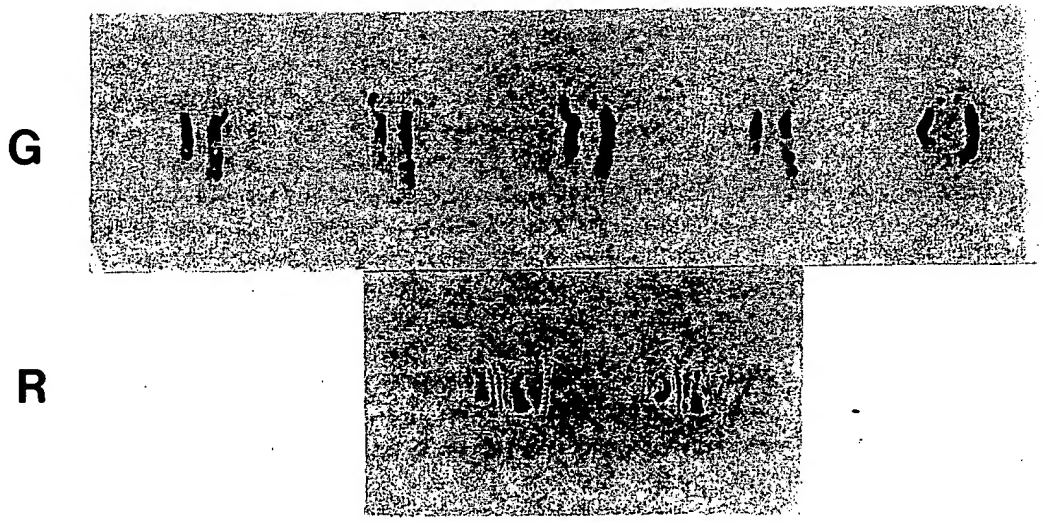

Figure 2 Cytogenetic analysis of DUP21JS. In each of the six chromosome pairs the normal 21 is shown on the left, and the duplicated 21 is shown on the right. The remainder of the karyotype was normal.

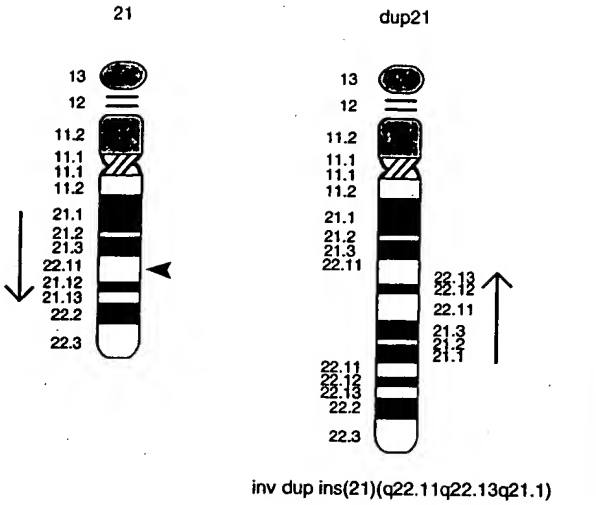

Figure 3 Ideogram of chromosomes 21 from DUP21JS that are shown in fig. 2. The arrows indicate the extent and orientation of the duplicated region. The arrowhead indicates the point of insertion.

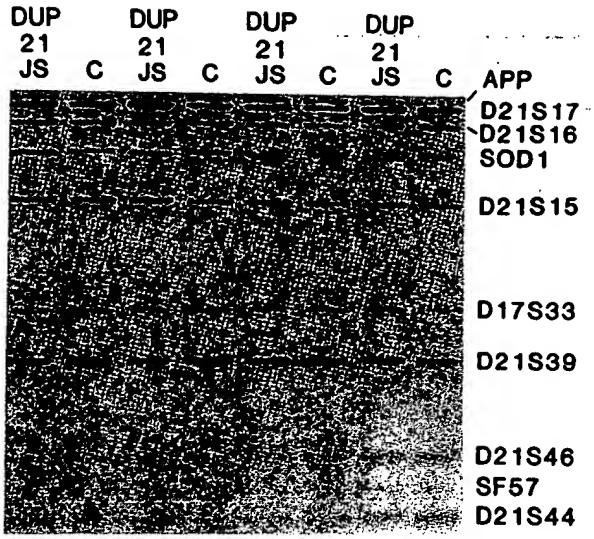

Figure 4 Representative autoradiograms from Southern blots used for analysis of DNA sequence copy number in DUP21JS and DUP21NA. Alternating lanes containing DNAs from patient DUP21JS and from the diploid control (C) are indicated. Autoradiographic bands corresponding to each DNA sequence are noted at the right of the figure. Bands corresponding to APP, *D21S17*, SOD1, and *D21S15* appear denser relative to those of the control (*D17S33*) and were present in three copies. This figure results from the superimposition of three autoradiograms, each resulting from one of three independent sequential hybridizations of the same membrane with three different sets of DNA probes. The densitometric analysis of copy number was conducted on individual autoradiograms whose bands fell in a largely linear range of film density.

Molecular Analysis

The *cytogenetic* overlap region defined by the two cases, DUP21JS and DUP21NA, is 21q22.1 (distal)-q22.2 and defines the region of chromosome 21 responsible for the shared clinical features of DS seen in these patients. To more precisely define both the extent of the regions duplicated and their molecular overlap, we made use of a physical map of this region. This physical map was constructed by using a somatic cell hybrid panel (Korenberg et al. 1990) and by quantitative Southern blot analysis in which a series of cell lines aneuploid for parts of a chromosome 21 were ordered by using the set of single-copy DNA probes specific for chromosome 21, as described in Material and Methods. We then used quantitative Southern blot dosage analysis and the DNA sequences shown in figure 5 to analyze the DNAs from DUP21JS and DUP21NA. A photograph of three superimposed representative autoradiograms used for the analysis of DUP21JS is shown in figure 4. This figure was generated as follows: A single Southern blot membrane was hybridized sequentially with three sets of probes. Autoradiograms for a given probe set were generated, and the signal was allowed to decay before the subsequent rehybridization. These individual autoradiograms were used for the densitometric analysis. However, the low background and precise band shape seen on the autoradiograms from each probe set and shown together in figure 4 permitted the superimposition of the resulting three autoradiograms. The relative band positions reflect molecular size within but not between each probe set, because of the displacement necessary to show each band clearly when superimposed. Regions that have been duplicated are present in three copies relative to the chromosome 17 reference probe (*D17S33*). Application of this technique showed that the duplication in patient DUP21JS extends from *APP* proximally through *D21S15* distally. Results of the statistical analysis for patient DUP21JS are shown in table 2 and are summarized in figure 5.

The molecular analysis for patient DUP21NA extended the previous study (Korenberg et al. 1990) and established that the duplicated region extends from *D21S55* through 21qter. The results of the statistical analysis are shown in table 2 and are summarized in figure 5. Because, on the basis of our physical map, the molecular regions of the chromosome that are present in three copies appear to be colinear in each patient, the alterations in both patients are probably simple chromosomal rearrangements.

Table 2

Copy Number in DUP21JS, for Chromosome 21-specific DNA Sequences

Probe	Ratio (95% CI)	Copy No.
D21S16	1.09** (.93, 1.27)	2
D21S46	.90** (.66, 1.23)	2
D21S1	.98*** (.88, 1.09)	2
D21S8	1.5	3
APP (FB68L)	1.54* (1.30, 1.83)	3
SOD1	1.42* (1.09, 1.56)	3
SF57	1.58* (1.37, 1.82)	3
D21S47	1.63* (1.41, 1.89)	3
D21S17	1.49* (1.38, 1.61)	3
D21S55	1.66* (1.52, 1.80)	3
D21S3	1.65* (1.38, 1.99)	3
D21S15	1.51* (1.32, 1.74)	3
D21S19	1.06*** (.95, 1.18)	2
BCE I	1.02*** (.91, 1.14)	2
D21S39	1.04** (.84, 1.28)	2
D21S44	.95** (.72, 1.26)	2

* $P < .001$ for hypothesis 1.0.
** $P < .01$ for hypothesis 1.5.
*** $P < .001$ for hypothesis 1.5.

Table 3

Copy Number in DUP21NA, for Chromosome 21-specific Sequences

Probe	Ratio (95% CI)	Copy No.
D21S47	1.16*** (1.01, 1.34)	2
D21S17	.80*** (.71, .90)	2
D21S55	1.50* (1.32, 1.70)	3
D21S3	1.62* (1.45, 1.81)	3

* $P < .001$ for hypothesis 1.0.
** $P < .01$ for hypothesis 1.5.
*** $P < .001$ for hypothesis 1.5.

Discussion

General Considerations

From a molecular analysis of patients with partial duplications of chromosome 21 and the features of DS, we have defined molecular markers for chromosomal regions likely to contain the genes for the congenital heart disease and DST seen in DS. Further, we have defined molecular markers for an overlap region that may be sufficient for the development of the facial and some other of the physical features of DS. In interpreting these results, it is important to consider the following: First, the definition of chromosomal regions is limited by cytogenetic resolution, and band definition may vary depending on the technique utilized. Second, a particular feature may be mapped by its presence more than by its absence, because, with the exception of neonatal hypotonia and mental retardation, no single DS feature is present in 100% of DS individuals with trisomy 21. However, despite this variability, map position is strongly suggested when a particular feature is (*a*) *present* at about the same frequency in full trisomy 21 as it is in patients with partial duplications of a specific region but (*b*) always *absent* when this specific region is not duplicated. For example, it is significant that congenital heart disease, present in about 40%–50% of DS, has been reported in two cases of partial duplications that include the distal region of q21q22 (Miyazaki et al. 1987; Korenberg et al. 1990) but has been reported in no cases (Park et al. 1987) that do not include the region of apparent cytogenetic overlap. As expected, CHD is also absent in two cases analyzed molecularly that include this region and in six further cases that do not include the region (McCormick et al. 1989; Rahmani et al. 1989; Korenberg et al. 1990).

Second, the more specific a feature is to DS, the more likely it is that a chromosomal region may be defined that contains the genes affecting its formation. This applies both to the endocardial cushion defects (ECD) characteristic of DS and to the DS DST discussed below. For example, about 70% of all ECDs are associated with DS (Ferencz et al. 1989). In contrast, less specific features found frequently in other aneuploidies of both 21 and other chromosomes, such as mental retardation, microcephaly, or the incurved

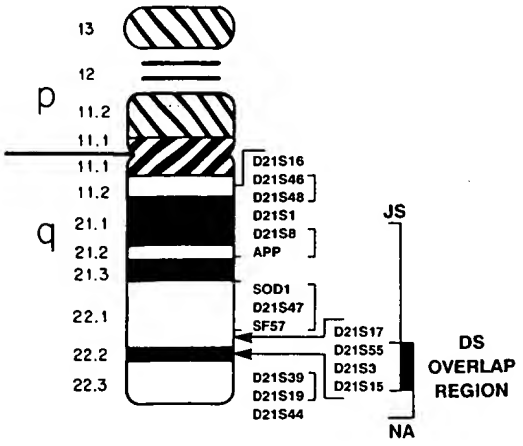

Figure 5 Chromosome 21 physical map of regions and DNA markers duplicated in DUP21JS and DUP21NA.

fifth finger, may be affected by genes in more than one region of chromosome 21. Nonetheless, although the individual facial features are each less specific than ECD or DST, they appear to be more associated with duplication of the q22.2 region. Finally, from individuals with partial duplication there is little information on other important features, including the risk of leukemia, Alzheimer-like dementia, and deficits of the immune system.

Our results shown in figure 5 suggest that DNA sequences present in both patients—i.e., the overlap region—include the chromosomal sequences defined by *D21S55, D21S15,* and *D21S3,* a region of about 3.7 Mb (Gardiner et al. 1990). The phenotypic features shared by the two patients include the facial features, wide space between the first and second toes, broad short hands, incurved fifth finger, and lax ligaments. Our data are consistent with the previous reports on the molecular analysis of two patients with partial trisomy 21, suggesting that duplication of either the *D21S55* region (Rahmani et al. 1989) or the region between *D21S58* and *D21S55* (McCormick et al. 1989) was involved in generating some of the facial, hand, and foot features of DS. The region responsible for some of the DS facial features has been variably localized, by both cytogenetic and molecular techniques, to the region in or adjacent to band 21q22.2 (McCormick et al. 1989; Rahmani et al. 1989; Korenberg et al. 1990; reviewed in Park et al. 1987). The high-resolution cytogenetic analysis of the overlap region reported here for DUP21JS and DUP21NA suggests that the most likely location of this region is either band q22.2 or the adjacent region in band q22.1. The difference between this assignment and that of Rahmani et al. (1989) (to band q22.3) reflects the limited resolution of cytogenetic analyses in such small chromosomal regions. This is due in large part to differences in the banding techniques utilized and because band appearances change with chromosomal rearrangement. Therefore, definitive mapping of the DNA sequences in the overlap region awaits the development of higher-resolution mapping methods on normal chromosomes.

It is important to note that chromosomal regions defined for the congenital heart disease and DST include the overlap region and may be either located within it or entirely separate. Therefore, the current data may *not* be taken as evidence for or against the existence of a cluster of genes responsible for all of the features of DS. Rather, it is clear from previous observations of mental retardation and non-DS dysmorphic features seen in individuals with more proximal duplications of chromosome 21 (revised in Epstein 1986 and Park et al. 1987) that genes in other regions of chromosome 21 must contribute significantly to the DS phenotype.

The overlap region defined here includes 2 of the 16 known chromosome 21–specific expressed genes (Hu ets-2 and ERG, an ets-related gene), in addition to 5 of the 22 known expressed DNA sequences unique to chromosome 21 (Gardiner et al. 1990). Clearly, these are now of particular interest with respect to their effects on growth and development and, when utilized for the generation of transgenic mice, may provide interesting information on the etiology of DS.

The DS phenotype-genotype map that results from these studies is shown in figure 6. This map was constructed by placing, within the molecular region shared, the phenotypic features shared by DUP21JS and the DUP21NA family. Other features are placed in the region of respective duplication. Therefore the map illustrates the minimal regions likely to contain the genes whose duplication is sufficient to cause a given feature. It does not exclude genes located in other regions from contributing to a particular phenotypic feature. For this, more extensive data on the lack of specific features are necessary.

Congenital Heart Disease

Our findings indicate that the region likely to contain the genes for congenital heart disease in DS extends from *D21S55* to the telomere—namely, the region duplicated in DUP21NA of the DS family. This region comprises approximately 9 Mb (Gardiner et al.

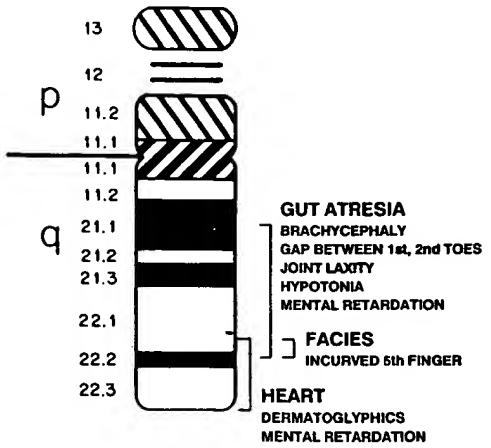

Figure 6 DS phenotypic map resulting from molecular analysis of DUP21JS and DUP21NA.

1990). Further narrowing of the region to the proximal half of this segment is suggested by combining a knowledge of the chromosome 21 physical map with data from the trisomy 16 (Ts16) mouse. This mouse has been the animal model of DS because mouse chromosome 16 (MMU 16) shares a large homologous region with human chromosome 21 (HSA 21) (reviewed in Epstein 1986). The Ts16 mice have a high incidence of congenital heart disease (Miyabara et al. 1982) similar to that seen in DS, suggesting that the genes in the MMU16 regions that are homologous to HSA 21 regions may be responsible. Preliminary data indicate that this region of homology includes only the proximal half of the region duplicated in DUP21NA and defined by *D21S55* through *MX1*, the MX influenza virus–resistance gene (Cheng et al. 1988; Reeves et al. 1989; Gardiner et al. 1990). Therefore these data, combined with ours, may narrow, to about 4–5 Mb, the region of chromosome 21 likely to contain the genes for DS congenital heart disease. This size of region may contain about 30 expressed genes, only a subset of which are expressed in the fetal heart. Therefore such studies may directly bridge the gap that allows the definition and testing of small numbers of genes responsible for heart disease.

DST

DST occurs in 1 in 10,000–40,000 live births but in 4%–7% of DS infants with full trisomy 21. Further, infants with DS constitute about 35% of infants with congenital DST (Lynn 1969). Consequently, although patients with DST and duplications of chromosome 21 are rare, definition of their duplicated regions may prove valuable in defining the genetic basis of DST. The duplicated region in DUP21JS provides a molecular definition of the regions of chromosome 21 that may be involved. This extends from *D21S8* through *D21S15*, a region of greater than 26 Mb. Although this is a large region, it is important to note that this excludes almost 40% of the known genes on chromosome 21.

The exstrophy of the bladder seen in DUP21JS is seen rarely in many chromosomal aneuploidies. It is neither specific nor common in DS and is therefore not placed on the phenotypic map.

Chromosome 21 Physical Map

Molecular analysis of the two cases presented in the present report further defines the physical map of chromosome 21. The inclusion of the DNA sequence *DS1S15* in the duplication of *DUP21JS* maps it distal to *D21S3* but proximal to *D21S39*, *D21S19*, *D21S44*, and *BCEI*, as shown in figure 4. Our cytogenetic analysis suggests that its possible map location is close to or within band 21q22.2. Further, the position of *D21S55* as distal to *D21S17* (Rahmani et al. 1989) is confirmed. The results presented in the present report begin to provide a basis for defining the molecular biology of both DS congenital heart disease and DS DST.

Acknowledgments

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Down syndrome phenotypes: The consequences of chromosomal imbalance

(chromosome 21/aneuploidy)

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ABSTRACT **Down syndrome (DS) is a major cause of mental retardation and congenital heart disease. Besides a characteristic set of facial and physical features, DS is associated with congenital anomalies of the gastrointestinal tract, an increased risk of leukemia, immune system defects, and an Alzheimer-like dementia. Moreover, DS is a model for the study of human aneuploidy. Although usually caused by the presence of an extra chromosome 21, subsets of the phenotypic features of DS may be caused by the duplication of small regions of the chromosome. The physical map of chromosome 21 allows the molecular definition of the regions duplicated in these rare cases of partial trisomy. As a first step in identifying the genes responsible for individual DS features and their pathophysiology, a panel of cell lines derived from 16 such individuals has been established and the molecular break points have been determined using fluorescence *in situ* hybridization and Southern blot dosage analysis of 32 markers unique to human chromosome 21. Combining this information with detailed clinical evaluations of these patients, we have now constructed a "phenotypic map" that includes 25 features and assigns regions of 2–20 megabases as likely to contain the genes responsible. This study provides evidence for a significant contribution of genes outside the *D21S55* region to the DS phenotypes, including the facies, microcephaly, short stature, hypotonia, abnormal dermatoglyphics, and mental retardation. This strongly suggests DS is a contiguous gene syndrome and augurs against a single DS chromosomal region responsible for most of the DS phenotypic features.**

Down syndrome (DS) is a major cause of mental retardation, congenital heart disease (CHD), and congenital anomalies of the gastrointestinal tract affecting the welfare of >300,000 individuals and their families in the U.S. alone. DS is also associated with a characteristic set of facial and physical features, defects of the immune and endocrine systems, an increased risk of leukemia, and an Alzheimer-like dementia; moreover, it is the prototype for the study of human aneuploidy.

From classical studies in plant genetics, pioneered by Blakeslee (1) in *Datura*, it was known that trisomy for chromosome arms produced easily recognizable phenotypes. When human trisomies were discovered, Patau (2) immediately planned to use partial trisomics as a way of mapping the diverse symptoms of these individuals. His earliest attempt is summarized in ref. 2. The present paper continues the theme, using the much more refined methods of modern cytology and molecular genetics.

With the discovery that DS was caused by trisomy 21 (3, 4), and the subsequent proposal that chromosome 21 band q22 was "pathogenetic" for DS (5), the foundation was laid for elucidating the fundamental biochemical and morphogenetic pathways of abnormal development in this aneuploidy. There followed a series of reports of individuals with "partial trisomy 21" (for review, see ref. 6) that appeared to indicate that regions might be defined that were likely to contain genes responsible for particular features of DS. These studies provide the basis for construction of a DS phenotypic map.

By "phenotype" we mean a measurable parameter and include clinical, physical, cellular, and physiological components. By "phenotypic mapping" we mean the molecular definition of a physical region that is likely to contain the gene(s) whose overexpression is ultimately responsible in part for the phenotype. The current revolution in human molecular genetics and the development of a physical map of chromosome 21 now provide the possibility to understand the genetic basis for some of these defects and, therefore, to provide a necessary first step for their prevention, amelioration, and perhaps ultimately, their treatment.

Phenotypic maps provide the basis for clinical prognosis for individuals with partial aneuploidy for chromosome 21, and when of high resolution, the basis for the identification of the genes responsible for the phenotypes. One approach to this combines the phenotypic information from individuals with "partial trisomy" such as those described above with a molecular definition of their duplicated chromosomal regions. Once the molecular markers for a region are defined, the genes within it may then be identified, characterized, and ultimately tested for their relationship to a given phenotype. This report describes the molecular and phenotypic definition of these individuals, provides a theoretical framework, and utilizes this to construct a molecular "map" of the phenotypes associated with DS.

MATERIALS AND METHODS

Two methods are used to define the regions duplicated in patients with partial aneuploidy for chromosome 21. These

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Abbreviations: DS, Down syndrome; FISH, fluorescence *in situ* hybridization; CHD, congenital heart disease.

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are quantitative Southern blot dosage analysis and fluorescence *in situ* hybridization (FISH). Each utilizes a series of previously mapped chromosome 21 DNA markers to define the copy number and/or structural rearrangement characterizing the aneuploid chromosome.

The approximate map positions and order for each of these loci is as indicated by physical mapping studies (7–10).

Procedures for DNA isolation and digestion, agarose gel construction, Southern blot analysis, probe labeling, hybridization, and autoradiogram development were conducted as described by Korenberg *et al.* (11). Southern blots utilized 8–12 paired lanes (16–24 lanes total) of patient and control DNAs. Densitometric analyses utilized the logarithmic transformation of density measurements. All probes were isolated as DNA fragments for Southern blot procedures or as plasmids or cosmids for FISH studies. The sources and references for all probes used can be found in *Human Gene Mapping 11* (12). DNAs were obtained from peripheral blood, fibroblasts, or lymphoblastoid cell lines. FISH studies were conducted with the techniques and procedures as described in Korenberg *et al.* (13).

Extended metaphase chromosome preparations were made from peripheral blood lymphocyte cultures by using methotrexate synchronization (14) and from skin fibroblast cultures. The chromosomes were stained by GTG-banding and reverse-banding techniques.

RESULTS

As a first step in establishing a "phenotypic map," a panel of individuals with partial duplications of chromosome 21 was assembled. By the DS protocols for clinical assessment established in Epstein *et al.* (6), the clinical features of 16 individuals with partial trisomy 21 were defined. Complete data were unavailable in many cases. All data were taken from the published literature or from the original records of the examining geneticist and were confirmed by follow-up examinations or discussions with the family and patient. The clinical evaluations are summarized in Table 1.

By using the Southern blot and FISH techniques, the chromosome 21 molecular content has been determined in the 16 cell lines derived from the individuals with partial trisomy 21. The results of the molecular studies are given in Table 2 and are summarized in Fig. 1.

Table 1. Clinical features of 16 patients with partial trisomies of chromosome 21

DUP21JG	DUP21GY	TETRA21MI	DUP21WB	DUP21JS	DUP21KJ	GM 0144	DUP21DS	DUP21SOS	GM 1413	DUP21SOL	DUP21JSB	DUP21NA	DUP21NO	DUP21BA	DUP21SM	Phenotypic Feature
+	+	-	-	+	-	+	±	–		±	-	+	+		+	Short stature
+	+	+	-	+	-		+	–		+	-	-	-		-	Microcephaly
-	+	+	-	+	+	-	-	–	+	+	-	-	-		-	Brachycephaly
-	-	-	+	+	+	+		+	+	+	+	+	+	+	+	Flat facies
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Up-slant palp. fissures
-	+	-	+	+	-	-		+	+	+	+	+	+	+	+	Epicanthic folds
-	+	-	-	-	-	-		+		-	+	-	-		-	Brushfield spots
	+	-	+	+	+	+		+		-	+	+	+	+	+	Flat nasal bridge
	-		+	-	+	-	+	–		+		-	-		+	Vaulted palate
	+	-			-	+	+	–	+	-					-	Furrowed tongue
	+	+		+	+	+	+	+		+	-	+	+	+	-	Open mouth
-	-	-	-		-	-	+	–	+	+	-	-	-		+	Malpositioned ears
-	-	-	-	-	-	+	+	–	+	+	+	-	-		+	Small/Dysmorph. ears
	+	+	+	-	-	+		+	+	+	-	-	-		+	Short neck
-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	+	Cardiac anomaly
-	-	-	-	+	-	-	-	+		-	-	-	-		-	Duodenal stenosis
-	-	+	+	+	±	+	+	+	+	+	+	+	+		+	Broad short hands
-	-	+	+	+	±	+	+	–	+	+	+	+	+		-	Brachydactyly
	+	–	+	+	±	+	+	–	+	+		+			-	Clinodactyly 5th finger
	±	–		+	+	+	+	+		-	+				-	Wide gap toes 1 & 2
	-	+			-		+		+			+	+		+	Abnl. dermatoglyphics
	-	+	–		-	+	+	–	+	-		+	+		+	Palmar crease
+	±	+	+	+	-			–		-	+	-	-	+	-	Hypotonia
+	-	+	+	+	+	+		+		-	+	+	+		-	Lax ligaments
P	59	M	M	M	M	43	P	M	37	P	M	42	P	P	52	IQ or MR (Moder. or Profound)

+, Presence of a feature; –, absence of feature; ±, borderline or marginal presence of feature; blank, no information was available. MR, mental retardation; P, profound MR; M, moderate MR.

DISCUSSION

It is useful to review the phenotypic features of DS as they provide a view of the potential of this approach for understanding the development of complex phenotypes. These are detailed in recent reviews (15, 16).

There are several important issues. (*i*) Trisomy 21 is associated with a rich variety of phenotypes. (*ii*) As seen in most autosomal dominant single-gene disorders, most of the phenotypic features are variable in both prevalence and expression. Two exceptions are the existence of mental retardation and neonatal hypotonia in close to 100% of individuals with DS. (*iii*) DS phenotypes may provide significant models for understanding development even when they are variable or of low frequency. For example, duodenal stenosis is seen in 4–7% of individuals with DS, but this accounts for 30–50% of all congenital duodenal stenosis (11). Moreover, although the DS endocardial cushion defects represent only ≈50% of DS CHD, individuals with DS account for close to 70% of all endocardial cushion defects (11). For both of these features, and quite likely for more, these data suggest the existence of gene(s) on chromosome 21 that are important in the development of the heart and gut both in DS and in normal individuals (11). Similar considerations suggest the existence of genes on chromosome 21 involved in the development of megakaryocytes (acute megakaryocytic leukemia) (17), the cornea (keratoconus) (for review, see ref. 18), aging (amyloid precursor protein) (for review, see ref. 19), and the brain (for review, see ref. 20). Although this last requires the more precise definition of specific phenotypes, an intriguing first candidate may be found in the investigation of the specific abnormalities of the brain-stem auditory-evoked potentials seen in DS. Because these are measurable at all ages, it may be possible to define small molecular regions containing fewer than 10 genes. Clearly, the potential relationship of such physiological "phenotypes" to the DS clinical cognitive abnormalities of auditory–verbal processing may be of significant interest for understanding a part of the mental retardation seen in DS.

There are many well-established potential sources for the phenotypic variability seen in full trisomy 21. This includes allelic heterogeneity for chromosome 21 (trisomic) genes, epistatic interactions (of chromosome 21 genes with genes on 21 or on other chromosomes), imprinting effects (variability of gene expression associated with the parental origin of the third chromosome 21), and environmental including stochastic and other pre- and postnatal events. These sources may clearly affect single-gene traits and the considerations of mechanism are similar. However, for individuals with partial aneuploidy, the chromosome structure is altered and the potential for position effects must also be considered, particularly for genes placed in close proximity to telomeres or to centromeres. For example, as in lower organisms, it is not unreasonable to expect that the expression of genes in a trisomic region or in regions bordering a deleted region may

Table 2. Results of the molecular studies on our eight patients with partial trisomy of chromosome 21

		JG		GY		WB		KJ		DS		SOL		JSB		SM	
Probe	Locus	Ratio	#	Ratio	#	Ratio	#	Ratio	#	Ratio	#	Ratio	#	Ratio	#	Ratio	#
pGSE9	D21S16	1.52	3	2.19	3	0.83	2			0.90	2			0.38	2		
pGSM21	D21S13E									1.04	2						
p21-4U	D21S110					1.06	2	1.14	2	0.82	2	1.10	2				
pPW228C	D21S1	1.30	3			0.82	2			1.49	3					1.20	2
pPW236B	D21S11																
pUT-B14	D21S116					1.87	3										
pPW245D	D21S8	1.39	3			1.70	3	1.18	2	1.46	3			0.86	2	0.69	2
FB68L	APP	1.53	3	2.36	3	2.20	3			1.45	3			1.19	2		
pUT-B79	D21S121																
JG108	D21S99		3f														
pPW513-5H	D21S54	1.18	2	1.33	3	1.35	3	0.80	2					0.84	2		
JG77	D21S93	1.13	2	0.88	2	1.12	2	1.79	3			1.08	2	1.11	2		
pUT-C43	D21S129	1.17	2			0.87	2					2.00	3	0.82	2		
pSOD1	SOD1	0.90	2f	0.80	2	1.10	2	1.39	3	1.64	3	2.60	3				
pPW524-5P	D21S58					1.64	3	1.59	3								
pPW525-5H	D21S65	0.93	2	0.99	2	1.90	3	1.40	3			1.40	3				
pGSH8	D21S17	1.02	2	0.89	2	1.54	3	1.90	3	1.26	3	1.90	3	1.05	2	0.93	2
pPW518-1R	D21S55	1.07	2			1.46	3f							1.88	3	1.31	3
V59	ERGB	0.86	2	0.82	2	1.39	3f							2.03	3		
pHO33	ETS2	0.98	2	0.93	2	0.82	2										
pPW231C	D21S3			0.96	2	0.76	2									1.68	3
pGSE8	D21S15	1.13	2	1.05	2	0.77	2	1.65	3	1.53	3	1.60	3			1.35	3
SF13a	D21S39	0.98	2	1.05	2	0.74	2			1.51	3			1.58	3	1.41	3
MX1a (1800)	MX1							1.09	2								
pS2	BCEI	0.95	2			1.08	2										
pGSB3	D21S19							1.02	2								
pGEM3	PFKL			1.15	2												
3.1.1	ITGB2			0.89	2	1.00	2										
SF50	D21S44	1.01	2	1.22	2	0.42	1			1.55	3			1.49	3		
pML1	COL6A1	0.80	2			0.58	1			1.99	3						
pML18	COL6A2											1.80	3				
pKN3	S100B																
pUT-B88	D21S123	1.00	2			0.50	1	1.07	2	1.42	3			1.65	3		

Ratio and copy number are given for Southern blot analyses. f, Independent test result by FISH study.

be decreased when rearrangement places them in regions of different chromatin structure such as centromeres, telomeres, or different bands. This apposition and the consequent potential change in expression may generate phenotypic variability unrelated to the genes in the aneuploid region. Such effects of chromatin environment on gene expression have not been demonstrated in humans but could be tested in this system.

The ultimate goal of constructing a phenotypic map is to define molecularly the chromosomal regions and ultimately the genes that are responsible for particular phenotypes. To do this, both the phenotypes and the molecular data must be well-defined. Although some individuals with small duplications exist, more often, the molecular data from many individuals must be combined to define small regions of 2–3 megabases that are suitable for molecular analysis. To combine data, one must consider the potential for both the phenotypic variability described above and for multiple sites affecting a single phenotype. When a trait is caused by the overexpression of a single gene or gene cluster, we may define the region containing that gene simply as the region of minimal molecular overlap of all individuals exhibiting the phenotype. However, if genes in more than one region contribute significantly to the phenotype, a simple overlap procedure may erroneously define the overlap region as containing the genes when, to the contrary, the gene(s) responsible are located in the nonoverlapped region. Therefore, it is important to determine which traits are caused largely by single genes or loci. To determine this, we should not ask which region is responsible, but rather, what part of the variability of a trait is contributed by the overexpression of genes in a given region. This question may be formulated in the classical genetic terms of penetrance, the probability of expressing a trait given the presence of the gene responsible, and expressivity, the variability of phenotypic expression of a trait, given that it is expressed.

The gene(s) in a single region may be largely responsible for a given phenotype when the penetrance and the expressivity of the trait are the same in individuals with full trisomy 21 and in individuals with duplications of the single region.

Then, as has been demonstrated, a simple overlap procedure may be used to create a phenotypic map. The expectation is that individuals whose duplication does not include the candidate region will not express the trait at a frequency above that seen in the normal population.

This supposition seems to hold true for the CHD seen in DS, in which the frequency of CHD in partially trisomic individuals varies with the region duplicated. In previous cytogenetic studies of individuals with duplications of regions outside of distal 21q22, 0% (0 out of 12 individuals) had CHD, whereas in 50% (9 out of 18) of those carrying duplications that included the region of distal 21q22.2–q22.3, CHD was evident (6, 11). Moreover, 33% (3 of 9 with CHD) had atrioventricular septal defects, similar to the proportion seen in full trisomy 21. Therefore, we may consider that the penetrance (percentage of DS with CHD) and expressivity (percentage of DS CHD that is atrioventricular septal defects) of CHD are similar in duplications of distal 21q22 and full trisomy 21. This suggests a single locus responsible for most of the variability of the trait.

By partitioning phenotypic variation in terms of penetrance and expressivity, our model allows for the definition of multiple-loci-affecting traits. For example, recent evidence suggests a possible role for *COL6A1* in generating DS CHDs (21). Such evidence would not necessitate a change in the map but could now be expressed as contributing a portion of the penetrance or expressivity of phenotypic variability, such as atrioventricular septal defects vs. atrial septal defects.

The DS phenotypic map, based on the 16 patients in this panel, is shown in Fig. 2. The gene candidate regions are defined and noted to reflect the possible contribution of one, two, or three and greater numbers of loci to the phenotype. This is to accommodate that, for less common traits in which the numbers of informative cases for any given phenotype become limiting, an analysis of penetrance and expressivity cannot be done. Moreover, in contrast to DS CHD, complete data for most phenotypes are lacking. Therefore, where only small numbers of informative cases exist, minimal regions are defined by the duplication observed and second sites must be considered. Finally, multiple genes must affect a trait when two nonoverlapping duplications exhibit the same phenotype. Where multiple nonoverlapping duplications are associated with a phenotype, each "island" of duplications may be analyzed separately.

Therefore, on the DS phenotypic map (Fig. 2), the following hierarchy of conventions is used:

If a single gene or cluster is responsible for most of the variability of a phenotype, the thick lines indicate its location. This minimal region is defined by the overlap of all contiguous cases manifesting the phenotype or by a single case, where only one exists. Where a single thick line is present, duplications including this minimal region generate phenotypes with the same penetrance and expressivity as seen in full trisomy 21; where there are two minimal regions, both would be necessary. However, for both single and double minimal regions, all cases with the phenotype must include at least one of these regions.

If two or more genes or clusters in a single island are responsible for a phenotype, the thin lines indicate the extent beyond the minimal region in which the two genes may be located. These are defined by the sum of the duplicated regions that have been independently associated with a phenotype. As for minimal regions, all cases with the phenotype would include at least one of these regions.

If three or more genes or clusters on chromosome 21 contribute to a phenotype, the dashed lines indicate the regions in

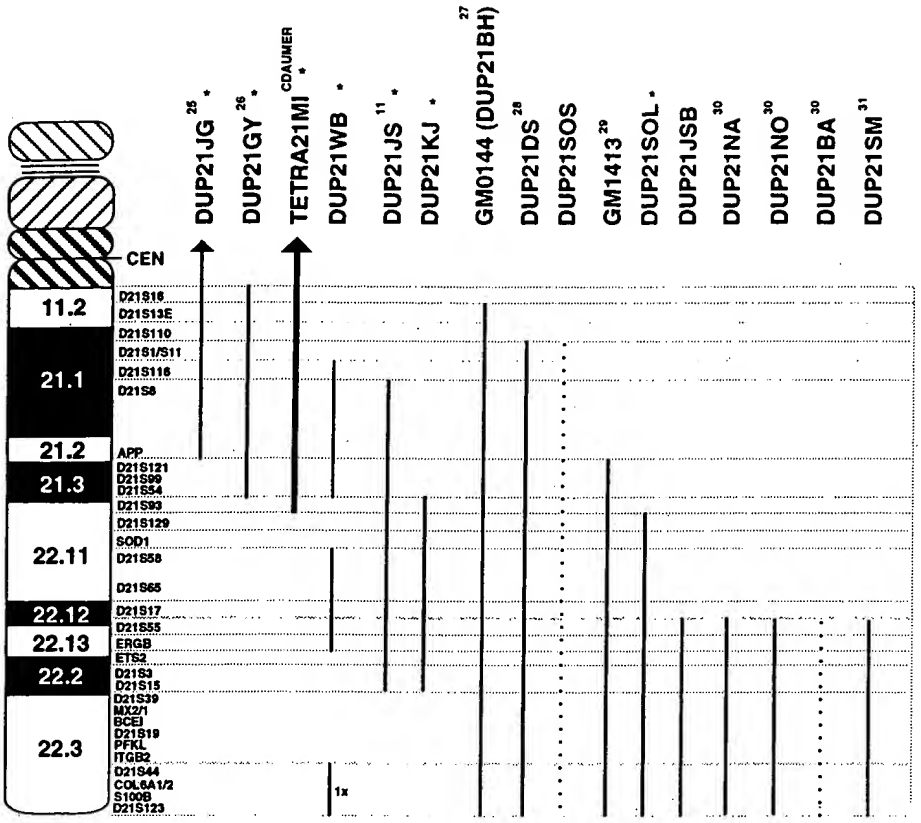

FIG. 1. Graphic summary of molecular studies on the 16 individuals used for the DS phenotypic map in Fig. 2. Solid lines, regions of duplication tested directly; dotted lines, findings inferred from family members with the same karyotype. Superscript indicates reference of published study; all other data have been collected by this laboratory except for data from TETRA21MI, which were from C.D., S.S., and J.R.K. (unpublished data). All cell lines are lymphoblastoid, except for GM 0144 and GM 1413, which are fibroblast lines obtained from the Human Genetic Mutant Cell Repository. *, Indicates aberration involving chromosome 21 only.

which the third may be located. These are defined by (usually large) duplications that have been associated with a phenotype but that include two nonoverlapping minimal or maximal regions, each of which must contain at least two genes.

The open regions have not been associated with the phenotype.

One significant conclusion from this map is that genes outside the *D21S55* region also contribute to what has been called the DS phenotype. This is based on the observations (Fig. 1 and Table 1) from three individuals with proximal trisomies that do not include this region but clearly exhibit typical DS features. It is not clear whether these features are

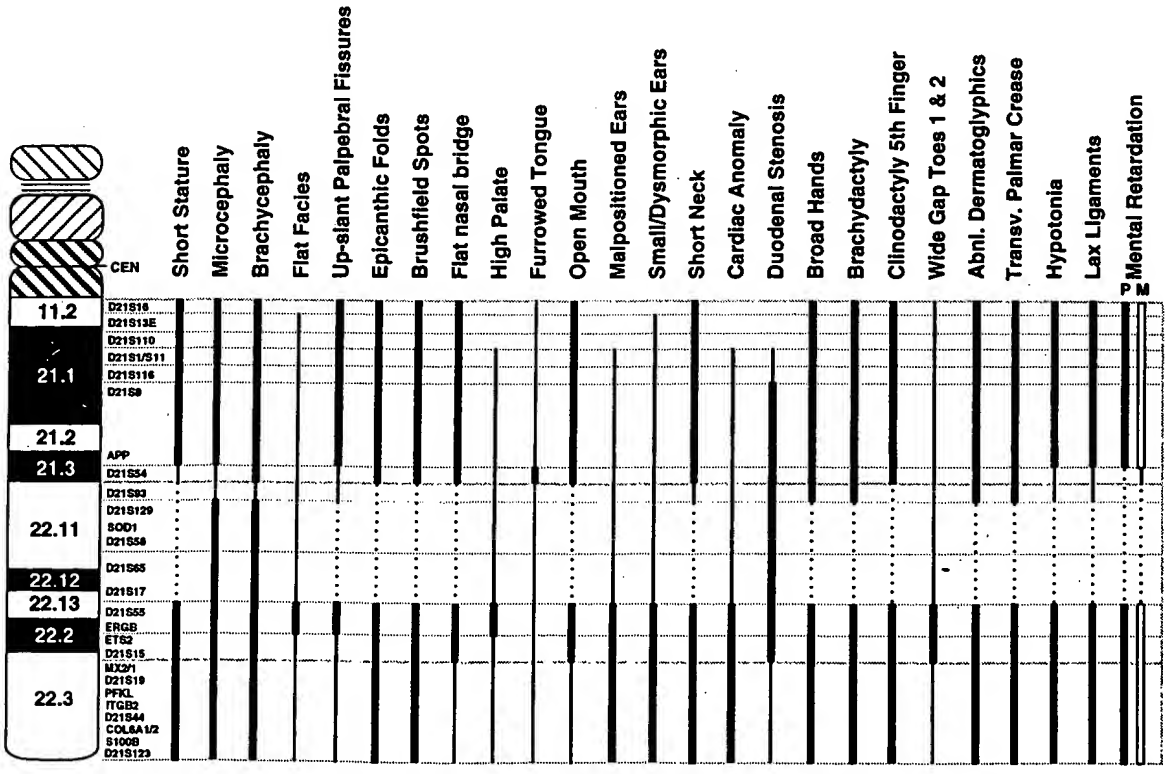

FIG. 2. Phenotypic map of 25 features associated with DS (see text for description and discussion of figure).

also affected by genes in other regions. However, the existence of second sites for many is expected in that all are somewhat nonspecific. In comparison to our model, Sinet and coworkers (22) utilize overlap exclusively to create the DS map. Because 9 of their 10 subjects include the region *D21S55*, it is difficult to conclude otherwise than this region is important in all of the features. Nonetheless, when viewed as minimal regions, their regions overlap ours for those features analyzed in common. However, the inclusion of only a single case with a non-*D21S55* duplication limits the evaluation of a DS chromosomal region and excludes the clear contribution of other regions to the DS phenotypes seen in our study.

A DS chromosomal region would imply that a single gene or gene cluster exists whose duplication is largely responsible for all DS features. From the DS phenotypic map data presented in Fig. 2, it is unlikely that such a region exists. We have shown that the duplication of regions distinct from distal 21q22 is sufficient to produce many of what have been called typical DS features. These second sites emphasize the necessity for an approach such as that detailed above for the construction of phenotypic maps. This would seem to suggest an alteration in nomenclature: away from the DS region and toward a more specific group of terms that associate particular regions with specific phenotypes, for example a DS CHD region or a DS gastrointestinal tract region.

Further indication for the role of multiple genes derives from the location of the chromosome 21 gene for amyloid precursor protein that is responsible for one type of familial Alzheimer disease, and possibly for the increased incidence of Alzheimer disease in DS. This is considerably distant from a region (*D21S55*–telomere) that is in part responsible for numerous other features including DS CHD (11). Moreover, although not yet shown, it has been suggested that the chromosome 21 gene for acute megakaryocytic leukemia associated with the 8;21 translocation (23) is also responsible for DS–leukemia risk. This gene is also found outside the region of *D21S55*–telomere. In contrast, we may still ask whether a subset of the DS phenotypes may be caused by or in some measure affected by the overexpression of a single gene or cluster. The current data from all sources are not adequate to resolve this question. In specific, there is still overlap between the regions defined for DS CHD, duodenal stenosis, and a part of the facial and other physical features, all of which could, in principle, be caused by a single gene.

Therefore, DS and its phenotypes are most accurately thought of as the result of the overexpression and subsequent interactions of a subset of the genes on chromosome 21. The DS phenotypic map thus reflects the nature of DS as a contiguous gene syndrome. Although usually reserved for syndromes caused by small deletions in which the genes are more readily defined, the term equally well represents the characteristic traits of DS.

Finally, while not directly related to DS, decreases in chromosome 21 gene copy number are under investigation (24) and may also shed light on underlying mechanisms leading to abnormal development. When the regions have been cloned in large fragment vectors such as yeast artificial chromosomes or bacterial artificial chromosomes, these reagents may be used to isolate and evaluate genes that are expressed in human (or mouse) embryonic tissues. For DS CHD, the entire region has been cloned in yeast artificial chromosomes, and cDNA libraries are being constructed from tissues obtained at this period of development.

All data suggest that we will be able to define the genetic basis of DS phenotypes and that this understanding will provide clues to understanding normal human embryonic development. Moreover, it will ultimately provide a basis for understanding and perhaps ultimately treating the associated defects, including CHD, gut disease, and some of the mental retardation.

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